Estimation and Occurrence of Select Antimicrobials in the Grand River Watershed

by

Nathanael Peter Couperus

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Author's Declaration

I hereby declare that I am the sole author of this thesis. This is a true copy of the thesis, including any required final revisions, as accepted by my examiners.

I understand that my thesis may be made electronically available to the public.

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Nathanael Peter Couperus

Abstract

Antimicrobials are pharmaceutically active compounds that destroy or inhibit the growth of bacteria, fungi, protozoa, or viruses. This diverse group of compounds, used in both humans and livestock, are increasingly being detected in the environment, especially in soil and aquatic ecosystems. Their widespread environmental occurrence is being linked to the potential development of resistance traits in microorganisms, which is a serious threat to global health. Quantifying this health risk is difficult due to the lack of available data on the consumption of antimicrobials, as well as the varying regulations on their use and distribution. Further, the environmental fate and occurrence of these chemicals in watersheds is complex and poorly understood. The current research aims to address this knowledge gap by examining the occurrence and distribution of select antimicrobials in a watershed through modeling and empirical data collection (survey). It achieves this goal by addressing the following objectives: (1) to develop a mass load model for estimating the residual concentrations of veterinary antimicrobials; and (2) evaluate the occurrence and sources of select antimicrobials in surface waters. The Grand River Watershed, a mixed-use watershed in Southern Ontario, Canada, was selected as the study site.

The mass load model was used to estimate the residual concentrations of four veterinary antimicrobials (lincomycin, monensin, oxytetracycline, and sulfamethazine) in the soil and water matrices. Predicted antimicrobial concentrations ranged from 0.1 μg/kg (monensin and oxytetracycline) to 60 μg/kg in soil (sulfamethazine) and 37 pg/L (oxytetracycline) to 18 μg/L (sulfamethazine) in surface water. Estimated antimicrobial concentrations were highest in sub-basins with high livestock densities, with the highest predicted levels found in the Nith sub-basin where there is intensive livestock production.

For the occurrence survey in the Grand River Watershed, triplicate water samples were collected from 27 sites in the main channel, one location each in five tributaries, and seven wastewater treatment plant (WWTP) effluents. Temporal sampling was also performed in six additional sites (four in an agricultural tributary and two in the main channel). The water samples were analyzed for five antimicrobials (sulfamethazine, sulfamethoxazole, trimethoprim, lincomycin, and monensin), and three chemical indicators (venlafaxine, ibuprofen, and atrazine). In the main channel, measured concentrations of target analytes exhibited an increasing trend from the headwaters to downstream towards the discharge point to Lake Erie. Peak concentrations measured in the river water were 98 ± 8.8 ng/L for antimicrobials (sulfamethazine) and 146 ± 67 ng/L for the indicators (ibuprofen). In the effluents, the highest measured concentrations were 355 ± 126 ng/L for sulfamethoxazole and 349 ± 11 ng/L for ibuprofen. Atrazine was found at low concentrations throughout the river samples but was not found in the wastewater effluents. Lincomycin was found in only a few samples and monensin was not found in any samples. Based on an analysis of the measured analyte concentrations, non-point sources were likely the main source of sulfamethazine in the main channel, while wastewater discharges were the main sources of sulfamethoxazole and trimethoprim. Sulfamethazine was detected at lower concentrations (8.8-65 ng/L) during the temporal sampling than during the large-scale sampling (peak concentration of 98 ± 8.8 ng/L), suggesting the significance of timing when collecting field samples for monitoring purposes.

Results from the model estimation suggest that livestock operations can be important sources of antimicrobials in receiving waters. Results of the survey sampling in the Grand River Watershed also suggest that agricultural sources and WWTP discharges are important sources of antimicrobials in the watershed. Over-all, none of the measured antimicrobial concentrations were above the 1 μg/L risk

threshold (for water) recommended by the Committee for Medicinal Products for Veterinary Use (CVMP). As tighter regulations on the use of antimicrobials emerge in Canada, and as more WWTP upgrades are completed in the Grand River Watershed, the findings of the current study can serve as baseline for determining the future impacts of these regulatory and infrastructure changes.

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Above all I would like to acknowledge my Lord and Saviour Jesus Christ without whom I can do nothing. He provides the inspiration for living. Soli Deo Gloria. To God Alone be the Glory.

Dedication

To Lindsay, the love of my life.

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Chapter 1: Introduction

This chapter provides a background to the research presented in this manuscript. It gives an overview of the consumption, environmental occurrence, and regulation of antimicrobials from global and Canadian perspectives. The chapter is organized into sections covering the following topics: 1) the global consumption of antimicrobials in livestock production and in human medication; 2) critical issues related to the widespread use of antimicrobials and the development of resistant traits in bacteria; 3) the environmental occurrence of veterinary antimicrobials as reported in literature; 4) recent global regulations on the control and consumption of antimicrobials, and 5) epilogue. The chapter concludes by defining the motivation, scope, and objectives for the current research.

1.1 Global Use and Consumption of Antimicrobials

Antimicrobials are pharmaceutically active compounds that destroy or inhibit the growth of bacteria, fungi, protozoa, or viruses. They are similar in function to antibiotics but are distinct from the latter because antibiotics do not fight against viruses (Kümmerer, 2009). Antimicrobials are ubiquitous in the environment largely due to their extensive use in livestock husbandry, in human medication, and in general antibacterial protection.

1.1.1 Administration in Livestock

In livestock farming, antimicrobials are administered to animals in four ways to reduce the risks of infections, decrease mortality, sustain growth, and increase production. These four treatments are: therapy, prophylaxis, metaphylaxis, and growth promotion. Therapy involves the treatment of infected livestock with antimicrobials until the infection is relieved. Prophylaxis is used as a control method for herds experiencing abnormally high death rates. Metaphylaxis is a preventative treatment for at-risk but disease-

free livestock. Finally, growth promotion is administered to prevent the onset of disease and to increase feed efficiency (Guardabassi et al., 2008; Kemper, 2008). Of these four types of treatment, growth promotion uses the most antimicrobials due to the longer duration of treatment and the larger livestock populations involved. In this treatment, an entire herd is dosed with low concentrations of antimicrobials, either through feed or water, to promote growth and nutrient assimilation in the body. When administered through feed, the antimicrobial concentrations are typically below 200 g/tonne of feed. To be considered a growth promoter, the antimicrobial must be administered for a period longer than fourteen days (Graham et al., 2007).

It has been argued that the increased use of antimicrobials in growth promotion has led to a subsequent increase in antimicrobial resistance (AMR) (McEwen, 2012; Witte, 2000). Antimicrobial growth promoters are also considered to be the main contributors to selective resistance in bacteria (Topp et al., 2012). Further, it has been suggested that international trading in the modern economy may induce the global transfer and development of AMR through the exportation and importation of animal products among countries (Maron et al., 2013; O'Brien, 2002). In this context, a country's efforts to control AMR through strict regulations on antimicrobials use may be undermined when contaminated products are imported from other countries without similar policies. Therefore, in formulating strategies for managing the spread of AMR, it is also important to consider national laws on antimicrobials use, per country consumption of antimicrobials, and the international trade flows of agricultural products (Cabello et al., 2013).

Monitoring the worldwide consumption of antimicrobials can be very challenging due to the large variability in the methodologies for data collection and synthesis among countries. Antimicrobials

consumption in the livestock industry is usually inferred from sales data or from estimates of livestock treatment doses (EMA, 2013). However, the available data can be inconsistent and often do not distinguish between imported and exported antimicrobials. The frequency of data reporting is also highly variable and, in general, very limited. The data shown in Table 1-1 were obtained by mining data in the database links listed by Page and Gautier (2012) and through literature search (covered period 2000-2014) in the Web of Science and Google Scholar using the combined keywords "antimicrobials", "antibiotics", "veterinary", "sales", and "country". As seen in Table 1-1, European countries report sales data on a more regular basis compared to the rest of the world. This systematic effort to consolidate sales data in Europe stems from the region's tighter regulations on the use of antimicrobials in their livestock industry. It is notable that data are unavailable for many countries in the larger and agriculture-dependent regions of Africa, Asia, Australia and Oceania, the Middle East, and South America. More importantly, consumption data, either from literature or from government agencies, are not readily available for most countries that are among the top livestock producers in the global market. Of the countries listed as top global producers of eleven livestock groups in Table 1-2, less than 15% have multi-year sales data (i.e., at least 2 years of reporting frequency) on veterinary antimicrobials since 2005 (Table 1-1). Eight countries are also the largest producers of at least five livestock groups; these are: China (9 groups), India (7 groups), Brazil (6 groups), Pakistan (6 groups), Ethiopia (5 groups), Mexico (5 groups), the Russian Federation (5 groups) and the United States (US, 5 groups). Of these countries, only the US currently reports antimicrobial consumption regularly in its livestock industry.

Table 1-1: Global Consumption of Veterinary Antimicrobials

Region	Country	Sales (Tonnes) by Year									Reference
		Average[a]	2005	2006	2007	2008	2009	2010	2011	2012	
Africa	South Africa[b]	1,538	-	-	-	-	-	-	-	-	Eagar et al., 2012
	Ethiopia[c]	416	-	-	-	-	-	-	-	-	Embassy of Ethiopia, 2012
	Kenya[d]	14.6	-	-	-	-	-	-	-	-	Mitema et al., 2001
Asia	China[e]	6,000[m]	-	-	96,810	-	-	-	-	-	Zhao et al., 2010; Hvistendahl, 2012
	Korea[f]	1,533	-	-	-	-	-	-	-	-	Kim et al., 2011
	Japan[g]	870	-	-	-	-	-	-	-	-	JVARM, 2013
Australia	Australia[h]	452[n]	-	655	572	580	482	644	-	-	JETACAR, 1999; APVMA, 2014
and Oceania	New Zealand[i]	-	53	63	56	53	56	-	-	-	MAF, 2011
Europe	Germany	-	-	-	-	-	-	-	1,819	1,708	EMA, 2013, 2014[j]
	Spain	-	-	-	-	-	-	1,746	1,779	1,693	EMA, 2013, 2014
	Italy	-	-	-	-	-	-	1,928	1,663	1,534	EMA, 2013, 2014
	France	-	1,322	1,260	1,346	1,188	1,064	997	896	762	EMA, 2011, 2013, 2014
	Poland	-	-	-	-	-	-	-	471	516	EMA, 2013, 2014
	United Kingdom	-	445	403	395	381	403	456	344	447	EMA, 2011, 2013, 2014
	Belgium	-	-	-	-	-	-	299	297	267	EMA, 2013, 2014
	Netherlands	-	508	544	589	525	514	461	363	246	EMA, 2011, 2013, 2014
	Hungary	-	-	-	-	-	-	206	147	179	EMA, 2013, 2014
	Portugal	-	-	-	-	-	-	181	164	157	EMA, 2013, 2014
	Denmark	-	111	114	119	117	129	119	106	107	EMA, 2011, 2013, 2014
	Ireland	-	-	-	-	-	-	96	87	100	EMA, 2013, 2014
	Switzerland	-	-	68	72	73	71	-	-	-	EMA, 2013, 2014
	Czech Republic	-	91	100	88	95	82	71	61	54	EMA, 2011, 2013, 2014
	Austria	-	-	-	-	-	-	63	53	53	EMA, 2013, 2014
	Cyprus	-	-	-	-	-	-	-	52	45	EMA, 2013, 2014
	Bulgaria	-	-	-	-	-	-	-	42	38	EMA, 2013, 2014
	Lithuania	-	-	-	-	-	-	16	14	13	EMA, 2013, 2014
	Finland	-	14	14	15	17	17	13	12	12	EMA, 2011, 2013, 2014
	Sweden	-	16	17	17	16	15	13	11	11	EMA, 2011, 2013, 2014
	Slovakia	-	-	-	-	-	-	-	11	10	EMA, 2013, 2014
	Estonia	-	-	-	-	-	-	7.6	7.5	7.3	EMA, 2013, 2014
	Norway	-	6.3	6.4	6.3	6.2	6.1	6.2	6.3	7.1	EMA, 2011, 2013, 2014
	Slovenia	-	-	-	-	-	-	8.4	7.8	6.8	EMA, 2013, 2014
	Latvia	-	-	-	-	-	-	6.6	6.0	6.7	EMA, 2013, 2014
	Luxembourg	-	-	-	-	-	-	-	-	2.2	EMA, 2014
	Iceland	-	-	-	-	-	-	0.9	0.7	0.7	EMA, 2013, 2014
Middle East	Iran	-	-	-	-	-	-	1,807	-	-	Aalipour et al., 2014
North America	United States	-	-	-	-	-	12,790	13,506	13,771	14,758	US FDA, 2010, 2011, 2012a, 2014[k]
	Canada	-	-	1,766	1,618	1,616	-	-	-	-	CIPARS, 2011
South America	Chile[l]	-	-	-	930	-	-	-	-	-	Cabello et al., 2013

a) Unless otherwise indicated, averaging period is unknown.

b) Yearly average: 2002 - 2004.

c) Value includes antibiotics, anthelmentics, and antiprotozoals, estimate is for 2012.

d) Annual mean quantities of antimicrobials administered to food producing animals between 1995-1999.

e) Prior to 2003, exact year not specified.

f) Prior to 2006, exact year not specified.

g) Yearly average: 2004-2010.

h) Data recorded from July 2005 to June 2010. Data in this table use the latter calendar year as reporting year (i.e. 2005/2006 data represented as 2006)

i) Data recorded from 1 April of current year to 31 March of following year.

Data in this table use the the latter calendar year as reporting year (i.e., 2005/2006 data represented as 2006).

j) EMA values are sales of antimicrobials for food producing animals and horses.

k) Includes domestic sales as well as export sales for food producing and non-food producing animals.

l) Includes only imported antimicrobials from tetracycline, florfenicol and quinolones classes, used mostly in salmon aquaculture.

m) Not specified if in short tons, long tons, or metric tonnes.

n) Yearly average of imported veterinary antimicrobials: 1992-1997.

Table 1-2: Top Global Producers of Livestock

Livestock Species	Total Head Produced 2011 (Millions)	Top 10 Producers (% of Total Production)	Percentage of Top 10 Producers Without Antimicrobial Use Data[a]
Buffalo	218.8	India (51.6); Pakistan (14.5); China (10.7); Nepal (2.3); Egypt (1.8); Myanmar (1.4); Philippines (1.4); Viet Nam (1.2); Thailand (0.7); Bangledesh (0.6)	100
Camels	26.8	Somalia (26.1); Sudan (17.6); Kenya (11.5); Niger (6.2); Chad (5.4); Mauritania (5.2); Pakistan (3.7); Ethiopia (3.7); Mali (3.5); India (1.6)	100
Cattle	1,586.8	Brazil (13.4); India (13.3); China (7.2); **USA (5.8)**; Ethiopia (3.3); Argentina (2.9); Sudan (2.6); Pakistan (2.2); Mexico (2.1); Australia (1.8)	89.4
Chickens	26,208.6	China (20.3); **USA (7.9)**; Indonesia (5.5); Brazil (4.8); India (3.6); Iran (3.4); Mexico (2.0); Russian Federation (1.6); Pakistan (1.3); Thailand (0.9)	84.6
Ducks	2,139.8	China (38.3); Viet Nam (4.5); Indonesia (2.3); Malaysia (2.3); Bangledesh (2.1); Thailand (1.5); **France (1.3)**; India (1.2); Russian Federation (1.2); Egypt (0.7)	97.7
Goats	1,166.8	China (15.9); India (13.5); Pakistan (5.3); Nigeria (4.9); Bangledesh (4.6); Sudan (3.8); Kenya (2.5); Iran (2.0); Ethiopia (1.9); Indonesia (1.5)	100
Horses	65.2	**USA (15.6)**; China (10.4); Mexico (9.8); Brazil (8.5); Argentina (5.5); Mongolia (3.2); Ethiopia (3.0); Kazakhstan (2.3); Russian Federation (2.1); Columbia (1.3)	74.7
Mules	13.1	Mexico (25.0); China (20.6); Brazil (9.7); Morocco (3.6); Ethiopia (2.8); Peru (2.3); Argentina (1.4); Iran (1.3); Pakistan (1.3); Columbia (1.1);	100
Pigs	1,439.1	China (32.7); **USA (4.6)**; Brazil (2.7); Viet Nam (1.9); **Germany (1.9)**; **Spain (1.8)**; Russian Federation (1.2); Mexico (1.1); **France (1.0)**; Poland (0.9)	81.3
Sheep	1,337.5	China (13.8); India (5.6); Australia (5.5); Sudan (3.9); Iran (3.7); Nigeria (2.8); **United Kingdom (2.4)**; **New Zealand (2.3)**; Pakistan (2.1); South Africa (1.8);	89.3
Turkeys	468.3	**USA (53.1)**; Chile (6.8); Brazil (5.8); **Italy (5.2)**; France (5.1); Russian Federation (3.6); **Germany (2.4)**; Morocco (2.1); Poland (1.8); **Portugal (1.4)**	28.9

a) Calculated as: Production from top ten producers who do not report multi-year antimicrobial use data (based on Table 1-1)/Total production from top ten producers.
Countries in bold font have available or accessible consumption or sales data for $\geq$ 2 years since 2005.
Source: Live Animal Statistics at: www.faostat.fao.org, as of 5 July 2014.

In addition to the variability in data reporting frequencies, there is also the issue of inconsistencies in data collection methodologies. For example, the Canadian data include sales of antimicrobials for food producing, sporting, and companion animals as well as fish stocks (CIPARS, 2011a). In contrast, European data include only the antimicrobials used in food-producing animals and horses (EMA, 2013). In general, reported sales data from most countries exclude antimicrobials used in farming aquatic stock such as salmon or shrimp (Cabello et al., 2013).

Standardized data collection methods and frequencies are critical in monitoring the global use of antimicrobials and its relationship with antimicrobial resistance. Consistent regional data aids researchers and policymakers in determining changes in the livestock population fluxes, trends in antimicrobial consumption, yearly variations in farming practices, and the effects of changes in legislation. Thus, it is important to have regular data collection to establish benchmarks and evaluate if strategies for curbing

antimicrobials use have the desired effects in controlling AMR. The European method for data collection and reporting is a good starting template for many countries. The consistency of data from European countries allows for the monitoring of antimicrobial consumption by class and livestock, and enables better identification of the critical antimicrobials that need to be monitored in relation to the control of antimicrobial resistance.

1.1.2 Administration in Humans

Penicillin and streptomycin were the first antimicrobials developed and administered to humans in the 1930s to fight against infections (Kumar et al., 2012). To date, there are approximately 3000 medicinal products with 250 pharmaceutically active compounds available for use as antimicrobials (Kumar et al., 2012; Kümmerer and Henninger, 2003). Each year, it is estimated that between 100,000 – 200,000 tonnes of these compounds are produced worldwide to treat humans and animals (Wise, 2002).

In most countries, antimicrobials for human medication can only be obtained through prescription; thus, the human consumption of antimicrobials has mostly been inferred from the number of dispensed prescriptions or sales data. Historical data on consumption is more readily available for the European region, which is also the first to regulate the use of certain antimicrobials in human and veterinary applications. Figure 1-1 shows the consumption of antimicrobials in Europe and in Canada in the period 2000-2009. The data are reported in units of defined daily doses (DDD), which is the average equivalent daily dose per patient. The European data are based on the number of outpatients while the Canadian data are based on sales. Except for a slight increase in 2003, antimicrobials consumption in Europe decreased from 2000 through 2004, and has since exhibited an increase until 2008. The Canadian sales also decreased through 2004, followed by an increase in 2006, and has since been approximately constant through 2009. There were no easily identifiable causes (e.g., infection epidemic, regulations) for the

fluctuations in use in Canada (Finley et al., 2013). In all years, the Canadian sales are below the average European consumption.

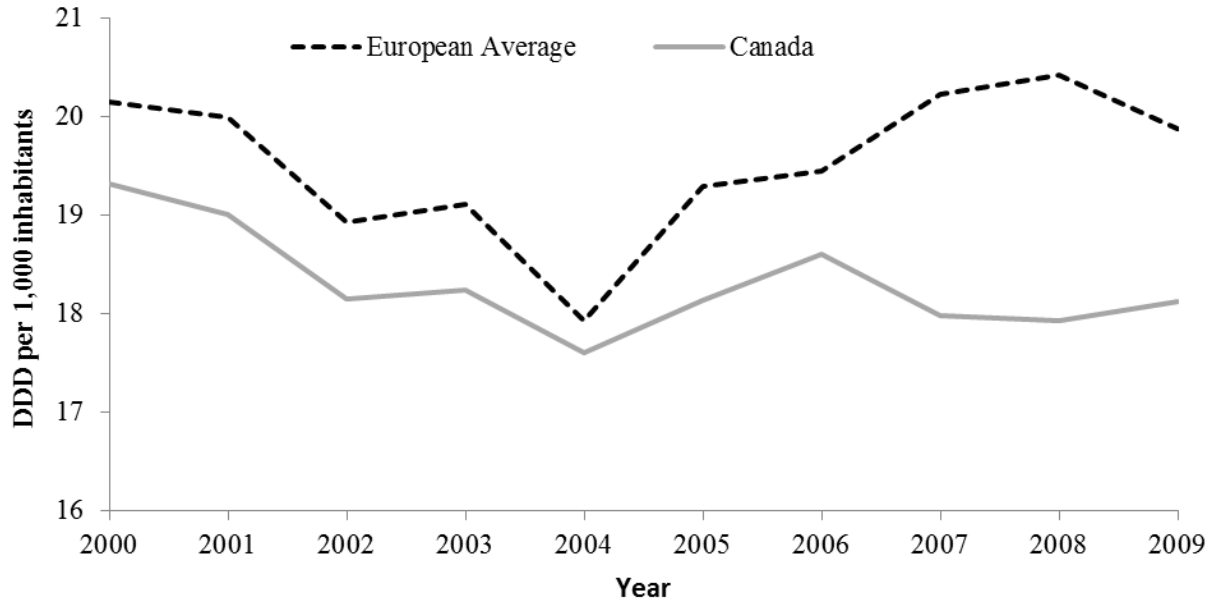


Figure 1-1: European Average Consumption and Canadian Sales of Antimicrobials per 1,000 Inhabitants from 2000-2009. Data were Compiled Following the J01 Classification (Antibacterials for Systemic Use) of the Anatomical Therapeutic Chemical Classification System Commended by the World Health Organization (CIPARS, 2011b; ESAC, 2009).

Recent data from Canada suggests that the human use of antimicrobials is considerably less than that in livestock production (Figure 1-2). From 2006-2009, human consumption (1600-1800 tonnes/year) accounts for approximately 10% of the total antimicrobials sold in Canada. This value is significantly less than estimates from previous researchers (50-50 split, Hughes and Heritage, 2004) The sales data for livestock production do not include antimicrobials brought into Canada through unregistered cross-border importation (discussed in Section 1.4), which is estimated to increase antimicrobials use in livestock by 25% (Prescott and Szkotnicki, 2012).

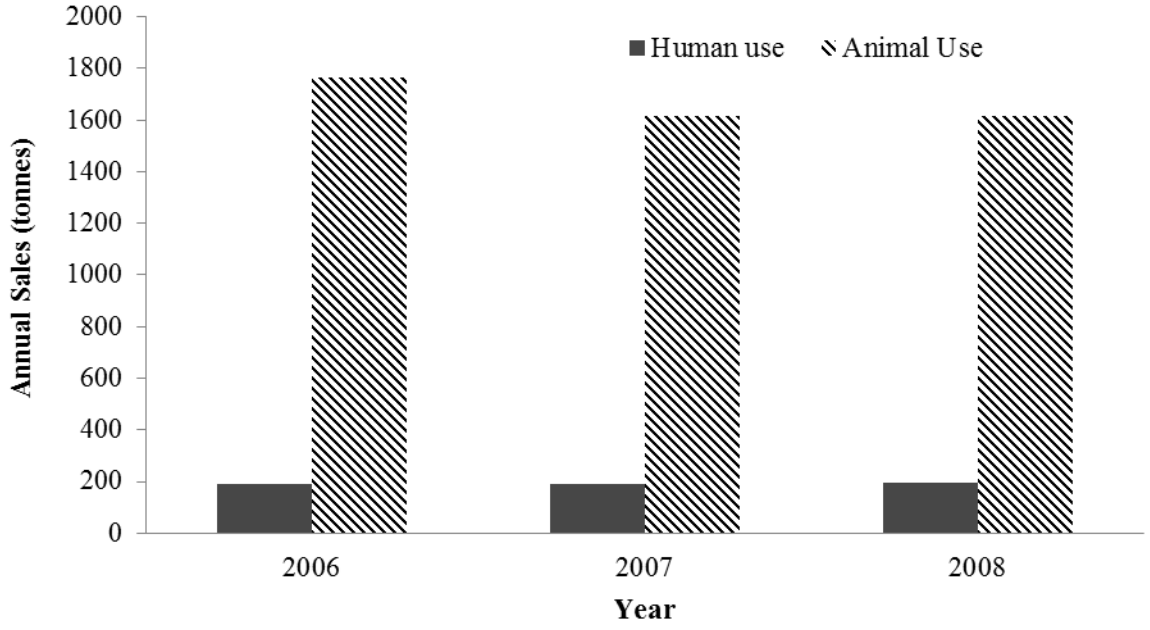


Figure 1-2: Canadian Antimicrobial Sales for Human and Animal Use as Reported by the Canadian Integrated Program for Antimicrobial Resistance between 2006-2008 (CIPARS, 2011a; CIPARS 2011b).

1.2 Antimicrobial Resistance

The global demand for meat products has increased in the last few decades, leading to a rise in animal production and antimicrobial administration. The annual per capita meat consumption rose from 24 kg in the mid-1960s to 36 kg in the late 1990s, and is estimated to rise again to 45 kg in 2030 (Bruinsma, 2003). Consequently, the densities of livestock in farms and the use of antimicrobials in animals have also increased to meet demand (Tilman et al., 2002). Researchers have raised concerns over the widespread use of veterinary antimicrobials due to the potential for developing AMR in bacteria (Alexander et al., 2008; Diarra et al., 2007; van den Bogaard and Stobberingh, 2000; Witte, 1998). Steadily feeding antimicrobials to animals at low doses may induce resistant traits in zoonotic and commensal bacteria (McEwen, 2012). The infected livestock can therefore act as reservoirs for resistant pathogens that can be transferred to

humans (Kemper, 2008). This can occur through cross-resistance, where a resistant strain develops resistance to an antimicrobial from the same class due to similarities in molecular structure, or through co-selection, where the same resistant gene has multiple resistant mutations that apply to multiple classes. To date, several laboratory and field studies have been performed to demonstrate the effects of antimicrobials exposure on bacteria. Increases in the prevalence of antibacterial-resistant populations have been observed in many cases (Vasquez et al., 2014; Gullberg et al., 2011; Liu et al., 2011; Costanzo et al., 2005).

The role of resistance transfer from companion animals (e.g., dogs and cats) to humans is not well understood but is a potential threat to human health (Jensen et al., 2008). The risk is attributed to the use of critical broad-spectrum antimicrobials in humans and in companion animals. For example, a 2003 study found methicillin-resistant *Staphylococcus aureus* (MRSA) in both a human patient and his dog, presenting a case of potential spread of the disease from the pet to the owner (Manian, 2003).

Resistant bacterial strains can also spread through the food chain, through direct contact with infected animals, and through indirect pathways in the environment. Quantifying the AMR transmission rates across these pathways can be challenging due to the high number of factors and variables involved in the process. The contribution of contaminated livestock manure and wastewater effluent to AMR transmission is not well understood because it is difficult to ascertain whether bacteria were already resistant before entering the natural environment or at what point in the AMR transmission chain the bacteria became resistant (Kümmerer, 2004). Despite this uncertainty, there is evidence in literature that suggests AMR development in human pathogens. A study by Finley et al., (2013a) found that human pathogens can acquire resistance genes through environmental exposure. Resistant bacteria, e.g., *E. coli*, have also been detected in drinking water sources and even in tap water (CIPARS, 2012a; CBC News, 2009). There is

also a marked rise in some cases of highly resistant pathogens, for example, MRSA rates in Canadian hospitals rose over ten-fold in the period 1995-2003 (Public Health Agency of Canada, 2005).

The study of AMR development and transmission in an ecosystem is a difficult undertaking due to the large number of variables involved and the complexity of the relationships among these variables. Generally there is a poor understanding of how bacterial resistance spreads in diverse ecosystems where multiple selective pressures are present (Cabello et al., 2013). A multi-variable AMR transmission study is resource-intensive and may involve the simultaneous determination of the rates of contact, transfer, integration, replication, diversification, and selection of resistant traits in bacteria and other organisms as they interact within a range of conditions (Martinez and Baquero, 2014). Monitoring these variables simultaneously can help identify specific factors that induce or inhibit AMR spread in the environment; however, this task is difficult to perform due to its complexity and cost. Despite this limitation, it is possible to infer AMR transmission trends from long-term and well-designed surveillance studies. Findings from a multi-year surveillance study of ceftiofur use in poultry have shown a strong correlation between ceftiofur and the development of ceftiofur-resistant strains of *E. coli* and *S. enterica* in humans (Dutil et al., 2010). The authors concluded that resistant bacterial strains that developed in chicken eggs were eventually transmitted to humans through retail chicken meat. They also suggested that this outcome could be attributed to the extra-label drug use (ELDU) for ceftiofur. Medication that is labeled ELDU can be used for treatment purposes other than those indicated on their prescription label.

The Dutil et al., (2010) study demonstrates cross-selection where the increased bacterial resistance to ceftiofur can potentially induce cross-resistance to other antimicrobials from the cephalosporin class that are commonly used to treat human infections. For this reason, the World Health Organization classifies ceftiofur as a Level I antimicrobial, or antimicrobials that are important in human medication (WHO,

2007). This classification was the basis for a recent decision by the US Food and Drug Administration (FDA) to ban the ELDU for cephalosporins (Prescott and Szkotnicki, 2012). In contrast, Health Canada only recommends avoiding ELDU labels for cephalosporins (Health Canada, 2008).

1.3 Environmental Occurrence

Residual antimicrobials in the environment are xenobiotic and thus can often be traced back to their sources. Point source contributions from wastewater treatment plant (WWTP) effluents are associated with prescription medication for humans. Non-point residual antimicrobials originate from agriculture, either through the discharge of contaminated wastewater, surface run-off, or through land application of contaminated manure (Yang et al., 2010; Christian et al., 2003). Accounting for non-point source contributions of antimicrobials can be challenging due to the high variability in their usage rates and dosages administered to livestock. Some agricultural practices (e.g., composting) can also compound tracking problems due to the transfer of antimicrobials between environmental matrices.

The majority of antimicrobial drugs are poorly metabolized in animals, and un-metabolized residuals are excreted in the feces and urine. For example, excretion rates for chlortetracycline, sulfamethazine and tylosin can range between 50% and 100% of their original doses (Sarmah et al., 2006; Thiele-Bruhn, 2003). Excreted antimicrobials can degrade, adsorb to soil, assimilate into plants, leach to groundwater, and run-off into surface water (Blackwell et al., 2007; Dolliver et al., 2007; Kreuzig and Höltge, 2005; Migliore et al., 1996). Literature data indicate that the environmental mobility of most antimicrobials is correlated with soil–compound interactions rather than with hydrophobicity alone (Tolls, 2001). Most antimicrobials bind strongly to soil and resist degradation (Kumar et al., 2005). Multiple studies have shown that detected concentrations of antimicrobials in surface water (ppt to ppb range) are significantly below minimum inhibitory concentrations for aquatic organisms (Wei et al., 2011; Boxall et al., 2005; Kolpin et al., 2002; Hirsch et al., 1999).

In Canada, despite the existence of intensive livestock production in large agricultural areas, there has not been any large-scale reconnaissance study for surface waters similar to that done by Kolpin et al. (2002) in the United States. The majority of environmental occurrence surveys of antimicrobials focus on wastewater effluents and antimicrobials for human medication. For example, Miao et al., (2004) surveyed eight WWTPs in five Canadian cities and frequently detected clarithromycin, erythromycin, roxithromycin, ciprofloxacin, ofloxacin, sulfamethoxazole, sulfapyridine, and tetracycline in the effluents. Guerra et al., (2014) also found human antimicrobials in five WWTP effluents over a two-year sampling period during summer and winter. Detected antimicrobials included azithromycin (210 ng/L), clarithromycin (1,100 ng/L), erythromicyn-H_2O (96 ng/L), ofloxacin (45 ng/L), and trimethoprim (170 ng/L). Human antimicrobials have also been detected in surface waters receiving WWTP effluents. Waiser et al., (2011) measured twelve antimicrobials including trimethoprim, sulfamethoxazole, amoxicillin, and triclosan at five locations along Wascana Creek in Saskatchewan. In this study, trimethoprim was detected as far as 60 km downstream from the WWTP.

Two Canadian studies have reported the detection of veterinary antimicrobials in surface water. Lissemore et al., (2006) conducted biweekly sampling over summer and spring at eight locations along the Grand River in Southern Ontario that have historically been susceptible to agricultural runoff. The antimicrobials frequently detected were lincomycin, monensin, and sulfamethazine at median concentrations of 12, 44, and 3.2 ng/L, respectively. Forrest et al., (2011) also found monensin, sulfamethazine, and salinomycin in water samples from twenty-three small watersheds that are impacted by agriculture runoff (< 1000 km^2) in the province of Alberta.

1.4 Regulations on Antimicrobial Use and Distribution

Strategies for curbing the spread and emergence of resistant pathogens vary considerably worldwide. Several countries such as Norway, Sweden, and Denmark have imposed complete or partial bans on antimicrobial growth promoters. Others have explored alternatives to antimicrobials such as natural growth promoters, probiotics, and in-feed enzymes (Hughes and Heritage, 2004). In 2011, the World Health Organization declared AMR as one of the top three threats to human health and urged countries to reduce antimicrobial use in veterinary practices and in human medication (WHO, 2011).

The 1969 Swann Report from the United Kingdom is one of the landmark comprehensive studies linking antimicrobials use and antimicrobials resistance in bacteria (Guardabassi et al., 2008). In the decades following the release of the Swann Report, European countries started the gradual removal of antimicrobial growth promoters in livestock feeds, beginning with tetracycline, penicillin, and streptomycin. Sweden first banned growth promoters in agriculture in 1986. Then Norway, Denmark, Germany, the United Kingdom, and other European countries began progressively imposing restrictions or bans on several antimicrobials for veterinary use. In 2006, a complete ban on antimicrobial growth promoters was enacted in the entire European Union (European Commission, 2003).

In the US, a wide range of antimicrobials, including several compounds that are considered important to human medicine (e.g., tetracylines and macrolides), are still permitted in veterinary practice as growth promoters. The US currently has the highest recorded domestic sales of veterinary antimicrobials for multiple years at approximately 13,700 tonnes per year (Table 1-1). It is estimated that growth promotion accounts for roughly 3 – 25 million pounds (1400 kg - 11, 300 kg) or approximately between 13% - 70% of the annual veterinary antimicrobial use in the country (Graham et al., 2007). More recently, the United

States Food and Drug Administration (FDA) expressed support for banning the use of antimicrobial growth promoters and restricting access to veterinary antimicrobials from over-the-counter access to prescription-only access (US FDA, 2012b). However, the removal of antimicrobial drugs in livestock feeds remains voluntary rather than mandatory (US FDA, 2013).

A few Asian countries have also enacted legislation for regulating the consumption of growth promoters in the livestock industry. The use of growth promoters in animal farms has been banned in Korea since 2012 (Kim et al., 2011). In Vietnam, a group of antimicrobial growth promoters, including chloramphenicol, is banned in agriculture (Kroismayr, 2007). In China, human health care policies have been amended in favour of prudent antimicrobial use but the country's Ministry of Agriculture has not imposed similar tighter restrictions for veterinary antimicrobials (Xiao et al., 2013). In the Philippines, therapeutic antimicrobials are permitted but many known antimicrobials used for growth promotion have therapeutic indications.

In Canada, Health Canada's Veterinary Drug Directorate has recently announced a three-year phase out plan for growth promoters and medically-important antimicrobials in the livestock industry (VDD, 2014). It has also imposed tighter oversight regulations over the use of veterinary antimicrobials. Prior to this development, approximately 90% of Canada's swine industry used antimicrobials (Kroismayr, 2007). The manufacture and sale of antimicrobials within the country is regulated through several federal legislations including the 1985 Food and Drugs Act (amended in June 2013), the 1985 Feeds Act (amended in June 2006), and the 1990 Health of Animals Act (amended in January 2013). The access to and use of antimicrobials are also regulated at the provincial level, with most provinces allowing over-the-counter (OTC) access to veterinary antimicrobials. Québec and more recently, Newfoundland and Labrador, are

the only provinces that restrict access to veterinary antimicrobials to prescription-only access. Current provincial legislation is not focused on controlling AMR but rather on limiting drug residues in food producing animals (Prescott et al., 2012).

Gaps in federal and provincial legislations give rise to two major loopholes in regulating the use and importation of antimicrobials in livestock production (Prescott and Szkotnicki, 2012). These loopholes are referred to as the own use importation (OUI) loophole and active pharmaceutical ingredients (API) loophole. In the OUI loophole, any Canadian can import up to a three-month supply of permitted drugs as long as they will be used solely for individual purposes and not sold commercially. This provision in legislation was originally intended to allow importation of drugs for human medication but it does not prohibit livestock producers from importing antimicrobials for use in herds (Prescott and Szkotnicki, 2012). Recently, the Ontario Medical Association advocated for tighter government control over cross-border importation of cheaper antimicrobials from the United States (OMA, 2013). The API loophole originates from the gap between the provincial and federal regulations on the use and sale of veterinary medicine. The provincial government regulates antimicrobial use and the federal government controls the sales of antimicrobials. Through this loophole, veterinarians and pharmacists can import bulk chemicals that are considered active pharmaceutical ingredients. Once in Canada, these chemicals can be mixed with other compounds for eventual use in medication, without oversight by Health Canada.

It is estimated that the OUI and API loopholes result in approximately $120 million annual loss in opportunity costs for the Canadian Government or the equivalent of 25% of current revenues from the sale of licensed veterinary drugs in Canada. Some of the suggested strategies to counter these loopholes include imposing bans on extra-label use of domestic and imported drugs and developing a new regulatory

framework for controlling the use of veterinary drugs based on their potential risk to the food supply and the end consumer. (Prescott and Szkotnicki, 2012)

1.5 Epilogue

This chapter underscores the increasing concern over the potential health threats associated with the extensive use of antimicrobials, the widespread environmental occurrence of residual antimicrobials, and the difficulties in regulating their global use. The emergence of resistant pathogens is a serious public health risk. Thus it is necessary to understand the eventual attenuation of antimicrobials in the environment.

For policymakers and water regulators, a thorough risk analysis is the first step in quantifying these health risks. However, given the persistence of antimicrobials and the limited data on their use, a more comprehensive understanding of their occurrence and fate in the environment is necessary to initiate risk assessment. Such large-scale studies are costly and time-consuming; they can also be challenging given the unregulated consumption of veterinary antimicrobials in many countries (Isaacson and Torrence, 2002). There are also some logistical challenges associated with the timing and scale of environmental sampling, which often requires skilled personnel and training in advanced instrumentation (Zhang, 2007). The timing of sample collection is crucial when monitoring environmental contaminants. Variables such as manure spreading practices and climatic variability need to be considered when designing sampling protocols and frequencies. The use of computational tools (e.g., models) can help simplify field sampling and reduce the over-all costs of risk analysis. For example, robust load estimation models can be utilized as a powerful screening tool for identifying priority compounds and locations that are vulnerable to residual antimicrobials. Identifying the sources, consumption trends, and residual loads of antimicrobials is critical

in mass transport modeling, which can be an integral part of risk analysis. Establishing consumption rates will also benefit efforts in assessing the health risks associated with the widespread use and occurrence of antimicrobials.

1.6 Research Objectives

The goal of the current research is to provide preliminary data on the occurrence and distribution of select antimicrobials in the environment through modeling and empirical data collection. The findings of this study can provide policymakers valuable insights on the consumption, sources, and environmental occurrence of relevant antimicrobials, and help identify strategies for mitigating their discharge and spread in the environment. The research results (model estimates and field data on antimicrobial concentrations) are also useful to other researchers who are interested in AMR risk assessment.

The specific objectives of the current research are as follows:

Objective 1: Develop a mass loading estimation model for veterinary antimicrobials to estimate their residual concentrations in a watershed; and

Objective 2: Examine the distribution of select antimicrobials in a mixed-use watershed.

The study area for this research is the Grand River Watershed (GRW), a mixed-use watershed in southern Ontario, Canada. Further description of the GRW can be found in Chapters 2 and 3. The rest of the research manuscript is organized as follows: Chapter 2 discusses the development and application of a mass load model for estimating the concentration of select antimicrobials in the GRW (Objective 1). Chapter 3 presents an analysis of the field data on antimicrobials that were collected from the GRW (Objective 2). Finally, Chapter 4 summarizes the findings and conclusions of this study, and identifies directions for future related work.

Chapter 2: Estimating Mass Loads of Veterinary Antimicrobials in Watersheds

This chapter discusses the development and application of a mass load model for estimating the concentrations of residual veterinary antimicrobials in the environment. The results of this chapter can provide critical information in the assessment of the potential risks associated with the high use of antimicrobials in the livestock industry, their disposal, and eventual environmental fate. The chapter is organized into four sections, namely: (i) introduction, (ii) development and validation of a mass load estimation model, (iii) case study, and (iv) conclusions and future work.

2.1 Introduction

The potential to induce bacterial resistance is the most serious health threat associated with the high use of antimicrobials worldwide. Estimates of global consumption indicate that on average, 50% of antimicrobials are used in agriculture (Hughes and Heritage, 2004). Other data suggests significantly higher consumption in countries with higher meat production, for example, it is estimated that 80% and 90% of the antimicrobials sold in the United States and Canada, respectively, are used in livestock (Palmer et al., 2011; CIPARS, 2011a). The high use of antimicrobials has been associated with the development of resistant genes in bacteria (Marshall and Levy, 2011). Similarities in resistance genes can give rise to resistance to multiple antimicrobials within the same class (cross-resistance). Resistance to a structural component of the antimicrobial can give rise to resistance in antimicrobials of other classes (co-resistance). Some bacterial resistance to human antimicrobials has been linked to the use of veterinary antimicrobials, for example, the veterinary use of avoparcin has been associated with the co-resistance of *Enterococcus faecium* to vancomycin, an antimicrobial used to treat serious infections in humans (Guardabassi and Kruse, 2008).

The frequent use of antimicrobials in livestock has also been linked to the widespread occurrence of low-level antimicrobials in the environment, which may induce resistant genes in pathogens and eventually intensify disease burdens in humans (Andersson and Hughes, 2012). Mitigating this health risk requires a comprehensive evaluation of the entry points for residual antimicrobials in various environmental matrices, an assessment of their eventual fate and toxicity, and a thorough understanding of the transmission pathways for resistant genes. However, such large-scale reconnaissance studies are resource-intensive and challenging to implement. Further, the difficulties in detecting low-level antimicrobials can be compounded by the temporal and spatial variabilities among regions (e.g., climatic conditions and varying farming practices). Despite these issues, it is widely recognized that the environmental prevalence of antimicrobials and resistant pathogens must be monitored to quantify their potential adverse impacts (Kümmerer, 2004).

A risk assessment is normally the first step in quantifying the environmental and health risks associated with emerging contaminants such as antimicrobials. This process involves identifying contaminant sources, estimating residual concentrations, and determining toxicity levels. The results of a preliminary risk assessment help regulators identify priority areas for further evaluation, and formulate strategies for mitigation. The assessment is usually carried out with the aid of simple models (e.g., mass load and transport models) and assumptions that enable the rapid quantification of the severity of the perceived risks (e.g., toxicity levels). With antimicrobials, one of the major challenges in risk assessment is the lack of available data on consumption and administration (see Chapter 1). A simple mass load model can be developed to address this issue by optimizing the use of limited data and inferring needed information from other surrogate data (e.g., livestock production). This model can be used to quantify residual concentrations, which in turn determine toxicity and exposure risks in indicator organisms.

In the present study, a simple mass load model has been developed to perform a rapid estimation of the levels of residual veterinary antimicrobials in soil and water matrices. The model was validated using data from previous studies and was applied to a case study of the Grand River Watershed in Southern Ontario, Canada. The environmental concentrations of four priority veterinary antimicrobials, namely, sulfamethazine, lincomycin, monensin, and oxytetracycline were estimated for each sub-catchment of the watershed.

2.2 Mass Loading Estimation Model Description

2.2.1 Predicted Concentrations in Soil and Water

The proposed mass load estimation model, shown in Equation 1, is based on the principles of mass balance. It integrates antimicrobial administration practices, livestock statistics, and the physical-chemical characteristics of antimicrobials to estimate residual concentrations in soil, which in turn is used to estimate residual levels in water matrices. The model is developed for the soil matrix on the premise that the most common entry point for veterinary antimicrobials in the environment is via land application of contaminated manure. With minor modification, the model can also be used to estimate the concentration of antimicrobials in manure.

$$\mathrm{PEC_S} = \left(\frac{\mathrm{D\ x\ T\ x\ B\ x\ L\ x\ F}_h \mathrm{\ x\ } F_e}{\rho_s \mathrm{\ x\ d}}\, x\, \frac{A_p}{A_f}\right) \mathrm{C} \qquad \text{Equation 1}$$

In Equation 1, PEC_s is the predicted antimicrobial concentration in soil amended with livestock manure (μg/kg), D is the daily dose of the antimicrobial administered to an animal (μg/kg/day), T is the duration of antimicrobial treatment (day), B is the average animal body weight (kg/animal), L is the livestock density in the pens (animal/ha), F_h is the fraction of the herd receiving antimicrobials treatment (value between 0 and 1), F_e is the fraction of the antimicrobial intake that is excreted in manure (value between 0 and 1), $\boldsymbol{\rho}_s$

is the soil bulk density (kg/m^3), d is the depth of manure penetration into soil (m), A_p is the area occupied by the pens (ha), A_f is the area where manure is applied (ha), and C is the units conversion factor.

The proposed model assumes that the livestock manure produced during the feeding period is entirely applied to land. A fraction multiplier can be easily introduced in Equation 1 if but a portion of this manure is instead used. Further, the model does not incorporate a spreading limit for manure, which is imposed in the European region to regulate nutrient inputs to farm soils. In Canada where the case application of the proposed model was performed (discussed in Section 2.3) an "individualized farm management plan" is enforced instead of a nutrient spreading limit (Robinson, 2006). In this plan, the amount of allowable manure that may be spread on a farm is predetermined from the characteristics of the underlying soil. For the case study, it is assumed that livestock manure is applied in the immediate vicinity of the livestock farm, either on the farmer's own lot or on adjacent lots. This assumption is made based on a Canadian study indicating that in practice, 15 km is the maximum distance at which it is economically sustainable to transport livestock manure for subsequent land application as fertilizer (Freeze and Sommerfeldt, 1985).

Lastly, the proposed model assumes negligible decomposition of the antimicrobials in manure before its amendment to soil. This assumption is conservative and can be easily modified to account for degradation during manure storage or treatment (e.g., composting). The rates of degradation for many antimicrobials during manure storage have reported to follow first order kinetics (Žižek et al., 2011; Wang and Yates, 2008; Schlüsener et al., 2006). These rates are influenced by many factors including the chemical properties of the antimicrobial, manure composition, and storage conditions (Kim et al., 2011; Ramaswamy et al., 2010).

The predicted antimicrobial concentrations in groundwater and surface water are calculated using Equations 2 and 3, respectively. These equations are recommended in the EU 93/67/EEC guidelines (EU

TGD 1998) and are used in this study without modifications. They are based on the partitioning behavior of contaminants at the soil-water interface under steady state conditions. Further, the equations assume negligible mass loss due to plant uptake or other biochemical transformations (e.g., photolysis).

$$PEC_{GW} = \frac{PEC_{s,20}\ x\ \rho_s}{K_{sw}\ x\ 1000} \quad \text{Equation 2}$$

$$PEC_{SW} = (\frac{\frac{PEC_s\ x\ \rho_s}{K_{sw}\ x\ 1000}}{3}) \quad \text{Equation 3}$$

In Equation 2, PEC_{GW} is the predicted antimicrobial groundwater concentration (µg/L), $PEC_{s,20}$ is the antimicrobial soil concentration at a manure mixing depth of 20 cm (µg/kg), and K_{SW} is the soil-water partitioning coefficient (µg/kg/ µg/L). In Equation 3, PEC_{SW} is the predicted antimicrobial surface water concentration (µg/L). This equation assumes that the antimicrobial in the manure partitions into pore water before it gets diluted threefold when it reaches the surface (EMEA 2008).

2.2.2 Model Validation

The proposed mass load estimation model in Equation 1 was validated using literature data for sulfamethazine and oxytetracycline consumption in swine and in cattle (Wang et al., 2014; Zhou et al., 2013). The predicted and measured concentrations are summarized in Table 2-1. In all cases, the predicted concentrations are higher than the measured concentrations, within the same order of magnitude and up to an order of magnitude higher. For comparison, predicted soil concentrations were also calculated using the EU Directive's CVMP equation (EMEA, 2008). Predictions from the daily normalized CVMP equation are higher than the measured antimicrobial concentrations by up to two orders of magnitude, and generally by an order of magnitude higher than the results from the proposed estimation model.

Table 2-1: Measured and Predicted Soil Concentrations

Literature-Reported Information			Prediction and Difference		Proposed Model Variables								CVMP Model Variables	
Antimicrobial	**Livestock Type**	**MEC_{soil}**	**PEC_{soil}**	**Δ**	**D**	**T**	**B**	**L**	**F_h**	**F_e**	**ρ_s**	**d**	**S_L**	**Ny**
Sulfamethazine	Swine	18.9 ± 6.4[a]	26.38[a]	7.5	11.5	1	106	25000	1	1	1150	0.1	1	1
		3.69 ± 0.42[b]	11.1[b]	7.4	10	29	106	500	1	1	1400	0.1	1	1
		18.9 ± 6.4[a]	527.7[c]	508.8	11.45	1	106	25000	1	-	1150	0.1	150	0.021
													1	
Oxytetracycline	Swine	36.8 ± 3.2[a]	109[a]	72.3	47.4	1	106	25000	1	1	1150	0.1	1	1
		36.8 ± 3.2[a]	35[c]	2.1	47.4	1	106	25000	1	-	1150	0.1	150	0.021
	Cattle	34.9 ± 1.0[a]	68.9[a]	34.0	307	3	200	430	1	1	1150	0.1	1	1
		34.9 ± 1.0[a]	9.32[c]	25.58	307	3	200	430	1	-	1150	0.1	150	0.049

Proposed Model Variables: D: Dose; T: Treatment Days; B: Body Weight; L: Livestock Density; F_h: Fraction Herd Treated; F_e: Fraction Excreted; ρ_s : Soil Density; d: Depth of Soil; S_L: Spreading Limit; N_v: Yearly Nitrogen Production

a) Wang et al., 2014
b) Zhou et al., 2013
c) CVMP Converted Daily

2.2.3 Sensitivity Analysis

A nominal range sensitivity analysis was performed to examine the sensitivity of the model predictions to the variables in the proposed mass load model in Equation 1. The analysis was carried out for the hypothetical scenario of swine livestock in Ontario, Canada receiving sulfamethazine in feedstuff. A base value for the predicted soil concentration, designated as PEC_S^0, was calculated using nominal values of the variables listed in Table 2-2, and considering a ratio of 1 for A_p:A_f (shed size same as farm lot size). The nominal values of the variables were the midpoint of their ranges. For each variable, the range of PEC_S values were calculated using the variable's high (x^+) and low (x^-) values that were either obtained or calculated from literature, and while keeping all other variables at their nominal values. For the sulfamethazine daily dose (D), the high and low values were calculated from CFIA (2014) and Wang et al. (2014). For the duration of treatment (T), the range was from the duration for stress therapy (10 days, CFIA, 2014) to rhinitis treatment (42 days, Bäckström et al., 1994). For the livestock body weight (*B*), the low value was the weight of a starter swine (12.5 kg) and the high value was the weight of a grower (65 kg) (CFIA, 2014). The livestock density was calculated as the number of swine divided by the available arable land in a region. Arable land includes land for crops, summer fallow, tame/seeded pasture and natural pastureland. The livestock densities for the Ottawa Division and Huron County were assigned as the low and high values (OMAFRA, 2011). For F_h (fraction of herd treated), a conservative value of 1.0 was assigned as the high value and 0.1 as the low value (for rhinitis treatment, Bäckström et al., 1994). The nominal value for F_e (excreted fraction of sulfamethazine intake) was the average of a conservative estimate (1.0) and 0.52 (Paulson et al., 1981). The soil bulk density ($\boldsymbol{\rho}_s$) ranged from 900 kg m^{-3} to 1600 kg m^{-3} (British Columbia Ministry of Agriculture and Food, 1990). The manure incorporation depth in soil (*d*) ranged from 0.05 m to 0.2m (EMEA, 2008).

Table 2-2: Nominal, High, and Low Values Used in Sensitivity Analysis

Variable	Units	Nominal (x^0)[a]	High (x^+)	Low (x^-)	Reference
Dose[b] (D)	µg/kg/day	192500	275000	110000	CFIA, 2014; Wang et al., 2014a
Treatment Duration (T)	day	26	42	10	CFIA, 2014; Bäckström et al., 1994
Body Weight (B)	kg/animal	38.75	65	12.5	CFIA, 2014
Livestock Density[c] (L)	animal/ha	1.07	2.1	0.043	OMAFRA, 2011
Fraction Treated (F_h)	Value from 0-1	0.55	1	0.1	Bäckström et al., 1994
Fraction Excreted (F_e)	Value from 0-1	0.76	1	0.52	Paulson et al., 1981
Soil Bulk Density (ρ_s)	kg/m^3	1250	1600	900	British Columbia Ministry of Agriculture and Food, 1990
Depth (d)	m	0.15	0.2	0.1	EMEA, 2008

a) The nominal value was taken to be the mean of the high and low x values.

b) Dose was calculated by multiplying dose (110mg and 44mg) by feed intake per day (2.5 kg).

c) Livestock density was found by dividing swine within county by arable land)

(Arable land is the summation of land for crops, summer fallow, tame/seeded pasture, and natural pastureland)

Figure 2-1 shows the effect of the variables on the predicted sulfamethazine soil concentration. For the range of variable values considered in Table 2-2, the livestock density (L) has the highest effect on the model predictions (nearly twice of PEC_S^0) while the soil density (ρ) has the least effect (28% of PEC_S^0), followed by the fraction of sulfamethazine excreted (F_e, 32% of PEC_S^0). This trend is largely due to the wide range of the L values, which vary by two orders of magnitude (10^{-2} to 10^0). These results suggest that for this case illustration, more careful consideration should be made in estimating livestock densities compared to the other variables in Equation 1.

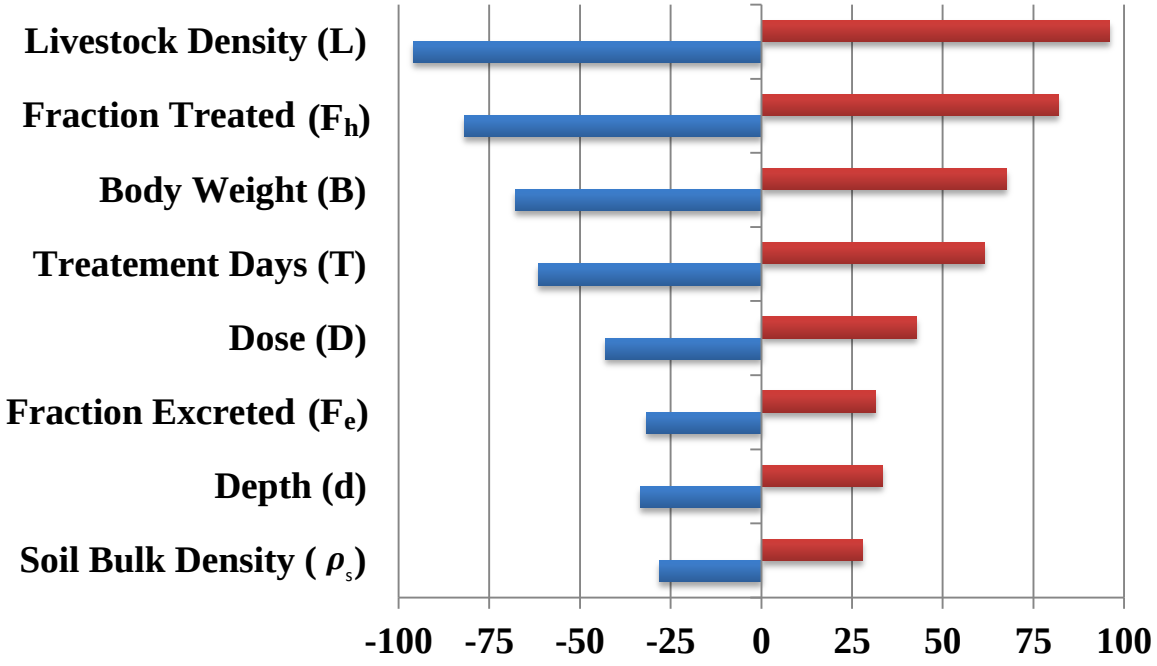


Figure 2-1: Effect of Variables on the Predicted Sulfamethazine Soil Concentration, PEC_S. The Livestock Density (L) has the Highest Effect on PEC_S (96% of base PEC_S) While Soil Density (ρ_s) has the Least Effect (28% of base PEC_S).

2.3 Case Study

2.3.1 Study Site

The proposed mass load model in Equation 1 was applied to a case study of select veterinary antimicrobials in the Grand River Watershed in Ontario, Canada. The watershed drains an area of 6800 km^2 and is the largest watershed in southern Ontario (Figure 2-2). It comprises 11 sub-basins: Upper Grand, Upper Middle Grand, Middle Grand, Lower Middle Grand, Lower Grand, Conestogo, Fairchild Creek, McKenzie Creek, Nith, Speed, and Whiteman's Creek. In addition to livestock production and crop farming, this mixed-use watershed supports manufacturing and commercial industries as well as urban development. Land is largely allocated to agriculture (75% is actively farmed) and intensive livestock production. In 2001, the watershed had an estimated 290,000 cattle, 500,000 swine, and 8.8 million poultry (GRCA, 2008). Livestock operations are heavily concentrated in the Upper Middle Grand, Conestogo, and Nith basins (Table 2-3). The majority of the population (985,000 in 2014) lives in the urban areas in the Middle Grand and Speed sub-basins (Chapman and Anderson, 2011; Farwell et al., 2008).

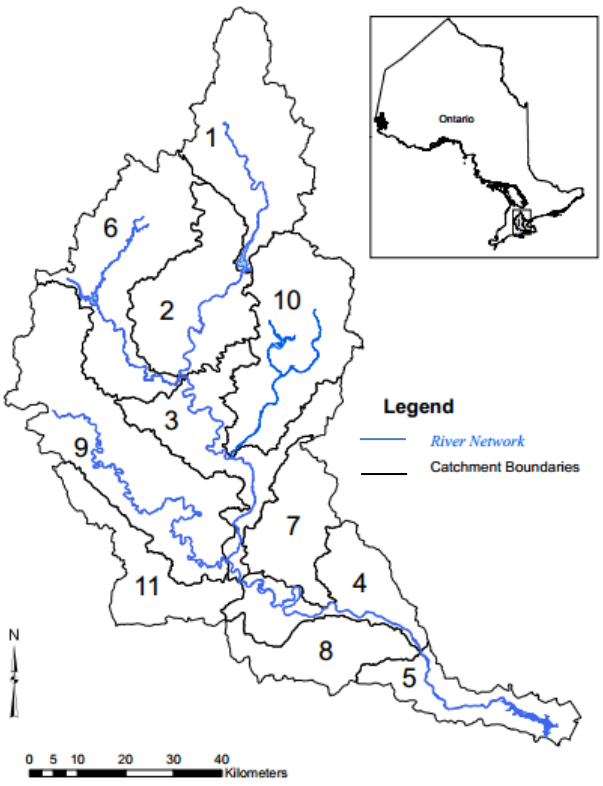


Figure 2-2: The Grand River (blue) and its 11 Sub-Basins (black): (1) Upper Grand, (2) Upper Middle Grand, (3) Middle Grand, (4) Lower Middle Grand, (5) Lower Grand, (6) Conestogo, (7) Fairchild Creek, (8) McKenzie Creek, (9) Nith, (10) Speed and (11) Whiteman's Creek.

Table 2-3: Livestock Density by Sub-basin (Livestock per Hectare)

Sub-basin	Density of Cattle	Density of Swine	Density of Poultry
Upper Grand	0.28	0.21	4.70
Upper Middle Grand	0.55	0.80	19.6
Middle Grand	0.56	0.63	16.6
Lower Middle Grand	0.11	0.11	7.90
Lower Grand	0.10	0.27	10.3
Conestogo	0.55	0.83	20.2
Fairchild Creek	0.17	0.14	9.30
McKenzie Creek	0.06	0.12	5.20
Nith	0.52	1.10	15.7
Speed	0.49	0.78	18.9
Whiteman's Creek	0.32	0.93	11.8

Sources: Livestock populations were calculated from data from OMAFRA (2011). Densities were calculated using averaging methods. Land area maps were provided by the University of Waterloo Geospatial Centre.

2.3.2 Data Inputs

The mass load model in Equation 1 was used to estimate the residual soil concentration of four antimicrobials, namely, sulfamethazine, lincomycin, monensin, and oxytetracycline, from three groups of livestock: swine, cattle, and poultry. These livestock groups were chosen because of their high production in Canada (Statistics Canada, 2014). The target compounds belong to different classes of veterinary antimicrobials and are among the most widely used antimicrobials in livestock production. Sulfamethazine is a sulphonamide used in the treatment of bacterial enteritis, dysentery and pneumonia in swine and in growth promotion in cattle. Lincomycin is a lincosamide used to treat mastitis in dairy cows as well as diarrhea in young swine. Monensin, an ionophore, is administered as a growth promoter in cattle and as a coccidiostat in poultry. Lastly, oxytetracycline is from the tetracycline class and is used to treat stress, enteritis, rhinitis, respiratory diseases and bacterial diarrhea in cattle, poultry and swine. In Canada in 2008, the combined sales of sulphonamides and trimethoprim amounted to 59 tonnes (for livestock use only). In the same year, 41 tonnes of lincomycin, 472 tonnes of ionophores, and 681 tonnes of tetracyclines were sold for veterinary use (CIPARS 2011a).

For the model application, the data inputs for each target antimicrobial and livestock are listed in Table 2-4. These values were either obtained or calculated from literature data. The estimated livestock densities and manure production in the Grand River Watershed are shown in Figure 2-3.

Table 2-4: Variable Inputs for PEC_S Calculations for the Case Study

Antimicrobial	Livestock Type	D[a]	T[a]	B	L[b]	Fh	Fe	ρ_s	d
		mg/kg	day	kg	animal/ha	0-1	0-1	kg m^3	m
Lincomycin	Swine	220	21	65	Table 2-3	0.42	0.21	1550	0.05
	Cattle	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	Poultry	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
Monensin	Swine	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	Cattle	0.000165	365	200	Table 2-3	0.76	0.95	1550	0.05
	Poultry	0.0033	365	1	Table 2-3	1.0	0.94	1550	0.05
Oxytetracycline	Swine	0.11	365	65	Table 2-3	0.15	1.0	1550	0.05
	Cattle	0.375	365	200	Table 2-3	0.1	1.0	1550	0.05
	Poultry	0.0038	365	1	Table 2-3	1.0	1.0	1550	0.05
Sulfamethazine	Swine	110	365	12.5	Table 2-3	0.08	1.0	1550	0.05
	Cattle	350	28	200	Table 2-3	0.05	0.3	1550	0.05
	Poultry	0.0038	365	1	Table 2-3	1.0	1.0	1550	0.05

Model Variables: D: Dose; T: Treatment Days; B: Body Weight; L: Livestock Density; F_h: Fraction Herd Treated; F_e: Fraction Excreted; ρ_s: Soil Density; d: Depth of Soil

N/A indicates that antimicrobial is not used in that particular livestock.
a. Dosage and treatment day information come from Compendium of Medicating Ingredient Brochures (CFIA) Accessed May 22, 2014.
b. Livestock density (L) values are different for each sub-basin. Values for each sub-basin for which the model was calculated can be viewed in Table 2-3.

2.3.3 Results and Discussion

2.3.3.1 Basin-wide Pattern of Predicted Concentrations

The predicted antimicrobial concentrations in soil and surface water are shown in Figures 2-4 and 2-5, respectively. Predicted soil concentrations range from 0.1 μg/kg (monensin and oxytetracycline) to 60 μg/kg (sulfamethazine). In general, predicted monensin concentrations are the lowest among the model estimates for the four target compounds, and are about ten to a hundred times lower than the predicted sulfamethazine concentrations (Figure 2-6; Table 2-5). Estimates for antimicrobial concentrations in

surface water range from the low ng/L to low μg/L levels. Model predictions for oxytetracycline, a compound that preferentially partitions to soil, are lower than monensin in surface water and ground water estimates and are generally four orders of magnitude lower than the predicted sulfamethazine concentrations.

Across the watershed, the trends in the predicted mass loads of antimicrobials generally follow the trend in livestock population and manure densities in the sub-basins (Figure 2-3). These trends indicate that the residual veterinary antimicrobials in the Grand River Watershed mostly come from the six sub-basins in the middle portion of the watershed. Estimated livestock densities and antimicrobial loads are highest in the Nith sub-basin, followed by the Whiteman's Creek, Conestogo, and Upper Middle Grand sub-basins. The Nith sub-basin has the highest predicted soil concentrations for sulfamethazine, lincomycin, and oxytetracycline at 60 μg/kg, 38 μg/kg, and 1.4 μg/kg, respectively. The predicted surface water concentrations for sulfamethazine (18 μg/L) and lincomycin (10 μg/L) are also highest in this sub-basin. The highest surface water concentrations for monensin occur in the Conestogo (0.84 μg/L) and Upper Middle Grand sub-basins (0.83 μg/L). The maximum estimated oxytetracycline surface water concentration is about a thousand times lower than predicted lincomycin concentrations, with a peak value of only 0.4 ng/L in the Nith, Conestogo, Speed, Middle and Upper Middle Grand sub-basins (Table 2-5).

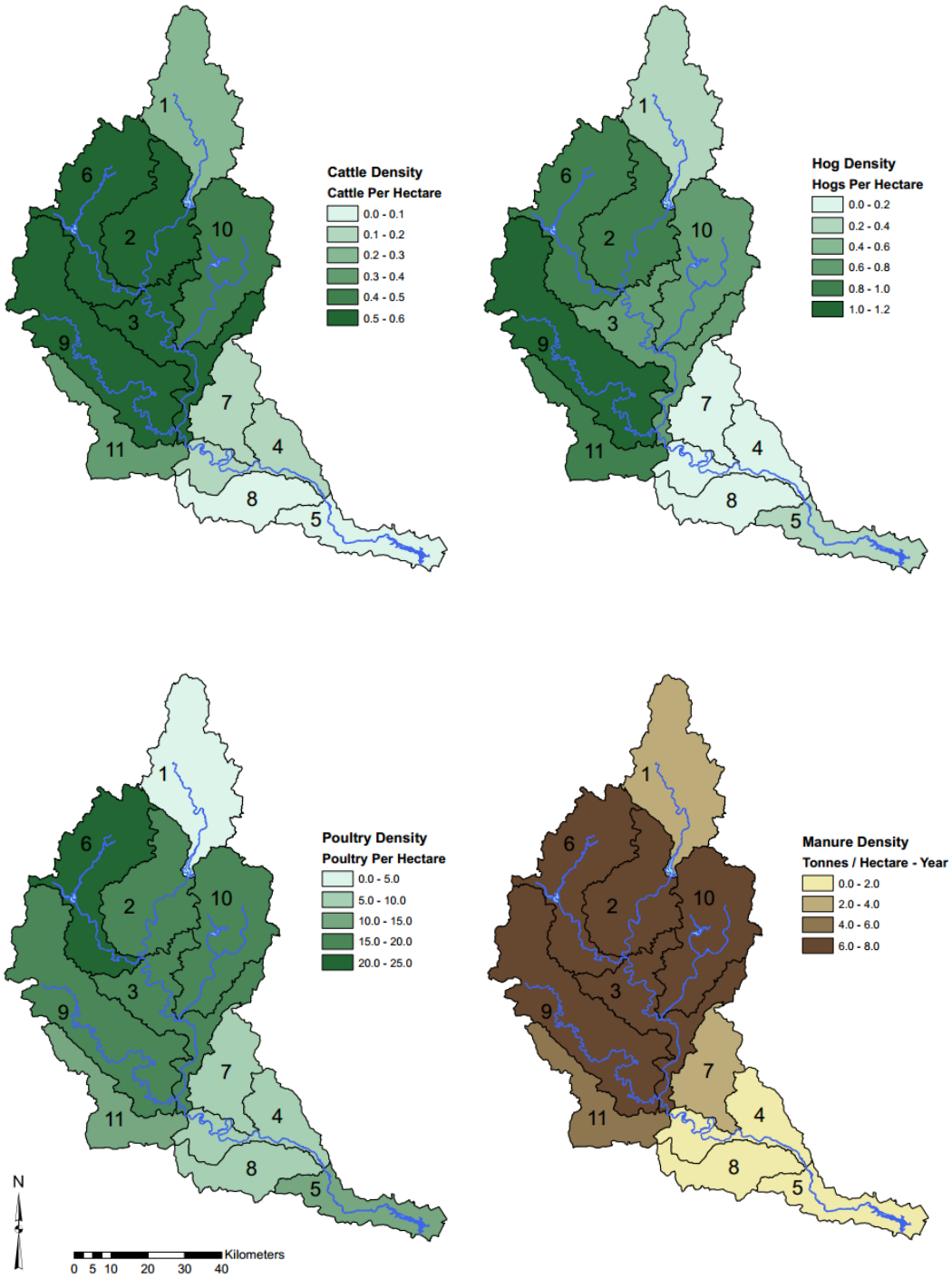


Figure 2-3: Estimated Density of Cattle, Swine, and Poultry Populations per Hectare as well as Total Estimated Manure Density in Tonnes per Hectare.

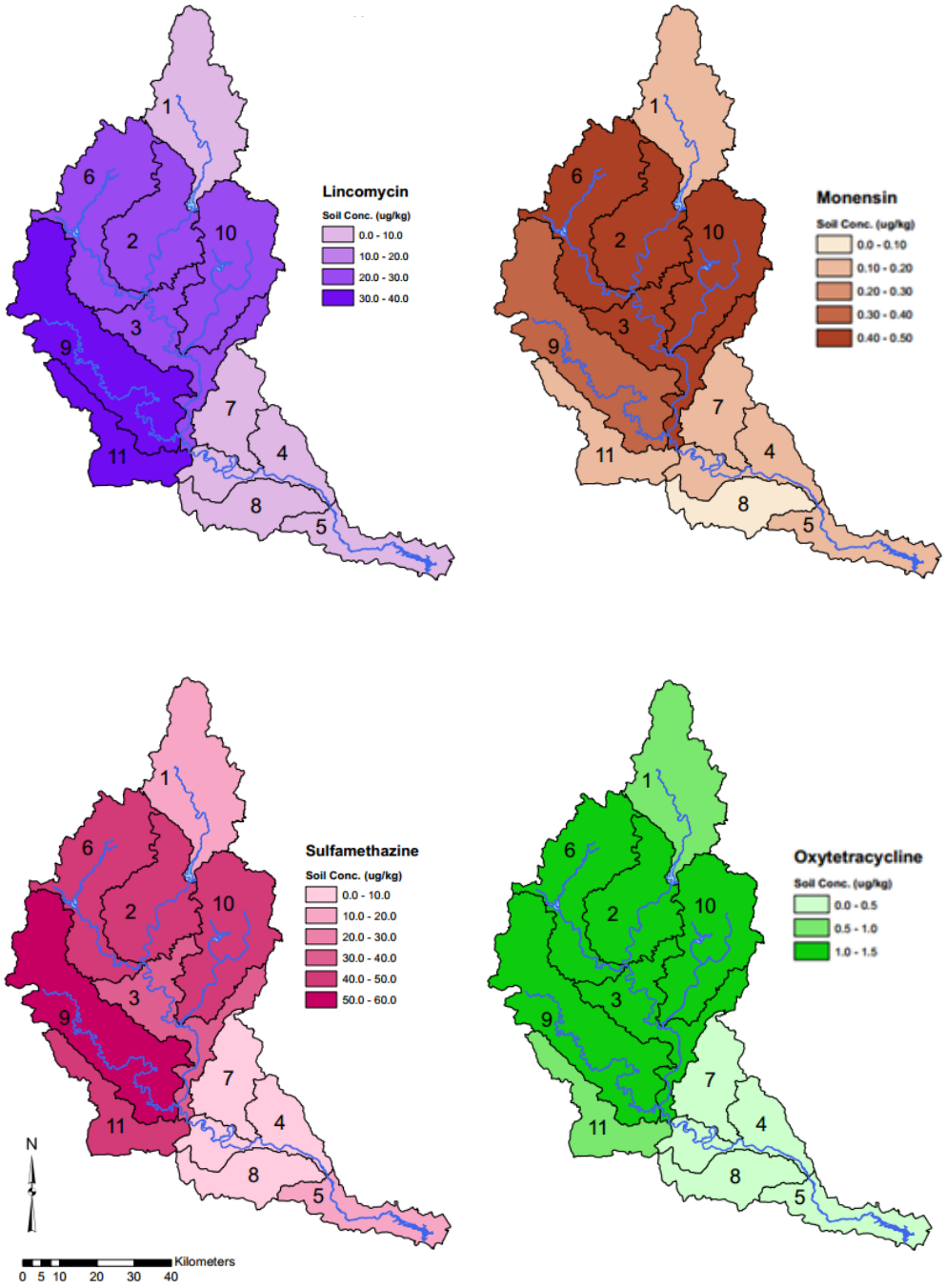


Figure 2-4: Predicted Soil Concentrations of Target Veterinary Antimicrobials in the Grand River Watershed

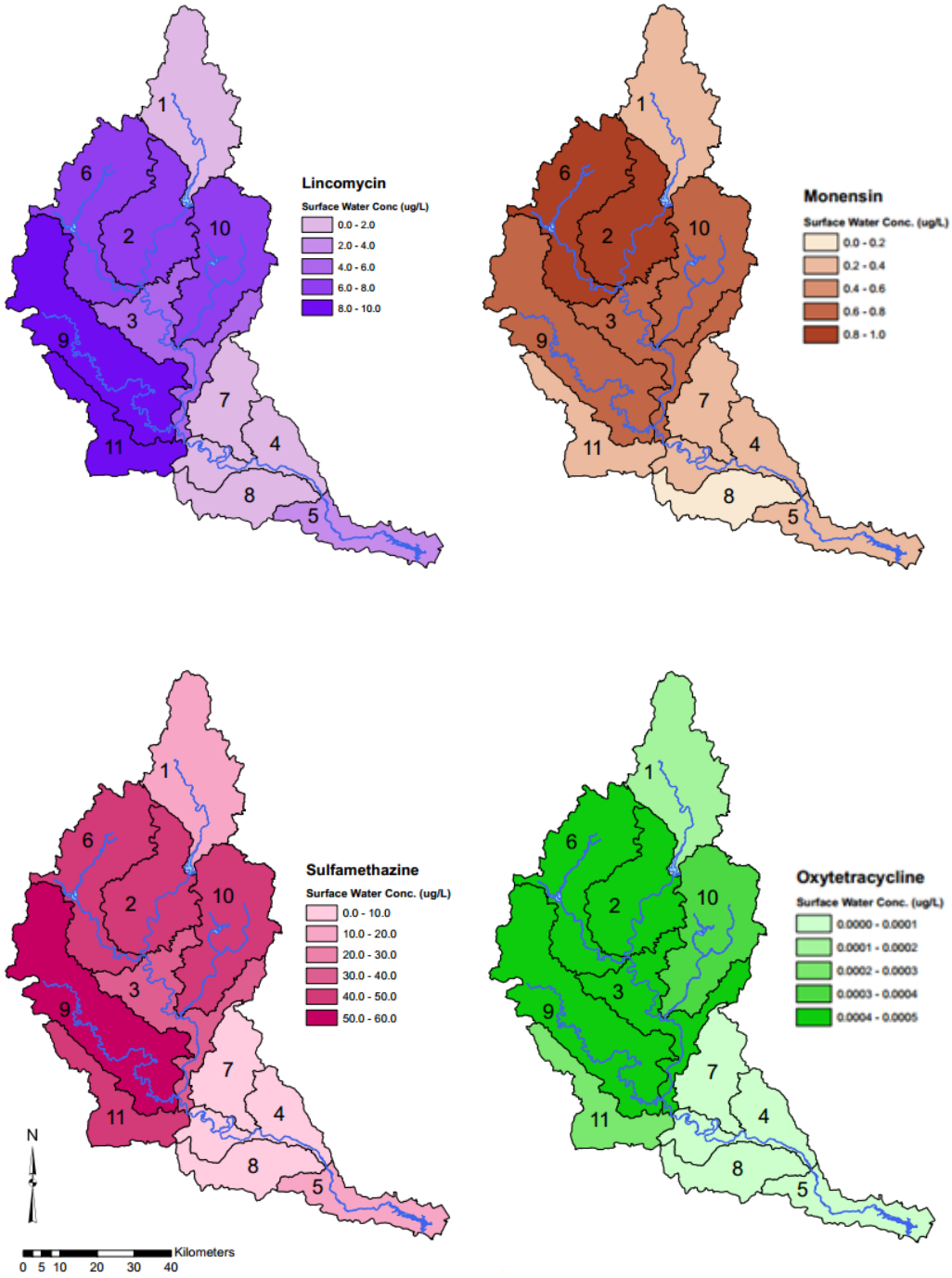


Figure 2-5: Predicted Surface Water Concentrations of Target Veterinary Antimicrobials in the Grand River Watershed.

Table 2-5: Predicted Environmental Concentrations of Target Antimicrobials in the Grand River Watershed

Sub-basin	PEC_S (µg/kg)				PEC_{SW} (µg/L)				PEC_{GW} (µg/L)			
	LIN	MON	OXY	SMZ	LIN	MON	OXY	SMZ	LIN	MON	OXY	SMZ
NITH RIVER	37.7	0.4	1.4	60	9.9	0.7	4.1E-04	18	7.4	0.5	1.8E-04	14
WHITEMANS CREEK	31.9	0.2	0.8	50	8.4	0.4	2.3E-04	15	6.3	0.3	1.4E-04	12
CONESTOGO RIVER	28.5	0.5	1.4	46	7.5	0.8	4.1E-04	14	5.6	0.6	1.5E-04	11
UPPER MIDDLE GRAND	27.4	0.5	1.4	44	7.2	0.8	4.1E-04	14	5.4	0.6	1.5E-04	10
SPEED RIVER	26.5	0.4	1.3	42	7	0.8	3.7E-04	13	5.2	0.6	1.4E-04	10
MIDDLE GRAND	21.3	0.4	1.4	35	5.6	0.8	4.1E-04	11	4.2	0.6	1.3E-04	8.2
LOWER GRAND	9.3	0.2	0.3	15	2.5	0.3	7.2E-05	4.5	1.8	0.2	4.4E-05	3.5
UPPER GRAND	7.3	0.1	0.6	12	1.9	0.3	1.7E-04	3.8	1.4	0.2	5.4E-05	2.9
FAIRCHILD CREEK	5	0.2	0.3	8.3	1.3	0.3	9.2E-05	2.6	1.0	0.2	3.6E-05	2.0
MCKENZIE CREEK	4.3	0.1	0.1	6.7	1.1	0.1	3.7E-05	2.1	0.8	0.1	2.1E-05	1.6
LOWER MIDDLE GRAND	3.6	0.1	0.2	6	1.1	0.2	5.2E-05	1.9	0.7	0.2	2.5E-05	1.4

The trends for the sulfamethazine, lincomycin and oxytetracycline concentrations follow the same pattern as the swine density map (i.e., highest concentration in the Nith). In contrast, monensin levels instead first peak in the Conestogo, Upper Middle, and Speed sub-basins, and then in the Nith sub-basin. This difference is attributed to the higher poultry densities in the first three basins. In Canada, monensin is prescribed only for cattle and poultry. Hence, despite the high swine density in the Nith sub-basin, swine manure has no contribution to the residual monensin load in the environment. In all environmental matrices (soil, surface water, groundwater), sulfamethazine has the highest predicted concentration, followed by lincomycin. In soil, the predicted oxytetracycline concentration is higher than the predicted monensin level; this trend is reversed in the water matrix where oxytetracycline has lower affinity.

The estimated soil concentrations of the four target antimicrobials are plotted in Figure 2-6. The top six contributors of antimicrobials, in decreasing order, are: Nith, Whiteman's Creek, Conestogo River, Upper Middle Grand, Speed River and Middle Grand. Individually, none of the target antimicrobials exceed the

recommended 100 µg/kg threshold for a Phase II toxicity risk assessment (EMEA 2008). However, the sum of the predicted antimicrobial concentrations in the Nith sub-basin is very close to this threshold value. Although the individual toxicities of antimicrobials have been reported for several indicator species, there are very few studies that have examined the synergistic or antagonistic effects of drug mixtures. Such studies are especially critical in studying the collective effects of antimicrobials on the development of resistant genes in bacteria (Vasquez et al., 2014).

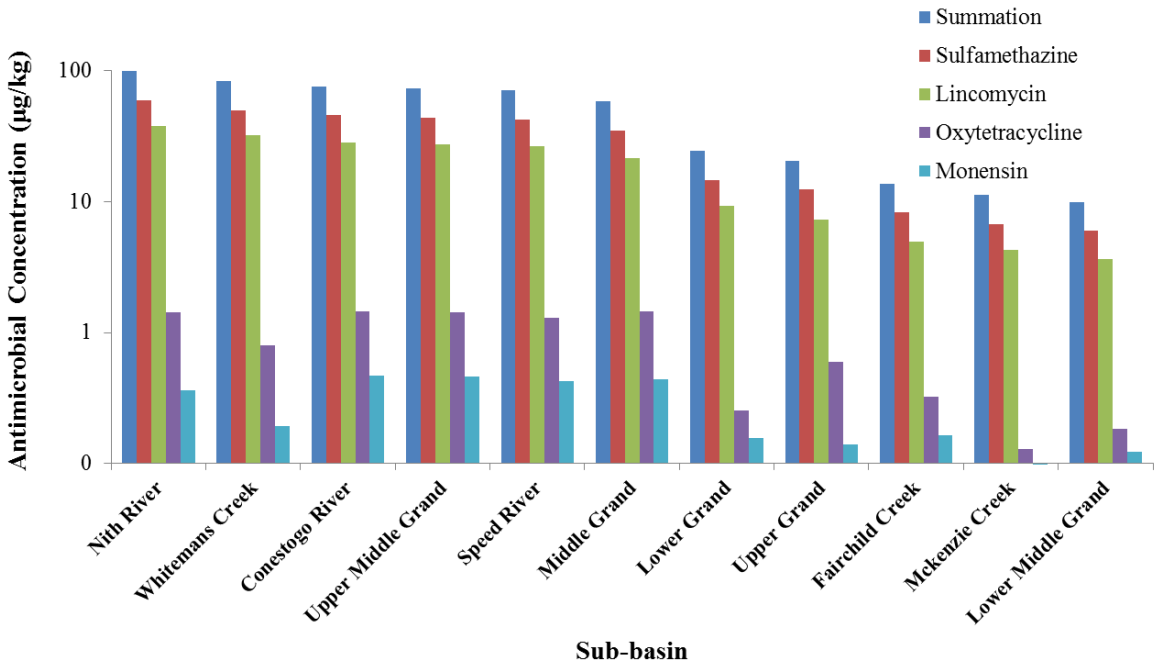


Figure 2-6: Estimated Soil Concentrations of Target Antimicrobials by Sub-basin

2.3.3.2 Correlations between Drugs, Animals, and Manure

Given the limited availability of accessible data on the consumption of veterinary antimicrobials (see Chapter I), manure production may be conveniently used as a surrogate indicator of the levels of antimicrobials in the terrestrial environment. To evaluate the validity of this approach, a multivariate correlation analysis was performed for the following parameters: target antimicrobial, livestock type, and

manure density. The analysis was performed using the Data Analysis toolkit in Microsoft Excel®. Of the four antimicrobials, three correlate well with swine density: sulfamethazine (R^2 = 0.96), lincomycin (R^2 = 0.99), and oxytetracycline (R^2 = 0.99). Monensin is most correlated with poultry density (R^2 = 0.99) while sulfamethazine is the only antimicrobial correlated with cattle density (R^2 = 0.96). Overall, manure density is well correlated with all of the four antimicrobials: sulfamethazine (R^2 = 0.99), oxytetracycline (R^2 = 0.94), lincomycin (R^2 = 0.92), and monensin (R^2 = 0.89). The order of these correlations reflects the impacts of the livestock size (or weight) on the antimicrobial mass load prediction. Swine and cattle produce significantly higher amounts of manure than poultry. Therefore, antimicrobials administered to larger animals may correlate better with manure production than those administered to smaller animals that excrete less manure. The results of the correlations suggest that the livestock type, livestock density, and the antimicrobial dosage are important in estimating the environmental concentrations of veterinary antimicrobials on a large scale. Results also suggest that manure production can be used to indicate the presence of certain but not all antimicrobials. For example, if there is a significant amount of manure from poultry, it is likely that monensin is present but not necessarily lincomycin.

2.4 Conclusions and Future Work

This chapter demonstrates the versatility of a simple mass load model in estimating the residual levels of veterinary antimicrobials in soil and water. The model requires only a few parameters, making it convenient to use especially when accessible data are limited. The case application for the Grand River Watershed suggests that residual concentrations of the studied antimicrobials do not exceed recommended threshold values for further toxicity risk assessment. The results from the case study could help risk assessors prioritize sub-basins and target compounds for further studies. The proposed estimation model can be used in conjunction with other contaminant transport models to study the attenuation of veterinary antimicrobials in small sub-basins or catchments. It can also be employed as a screening tool for assessing

the risks associated with the widespread use of antimicrobials that are used for therapeutic and non-therapeutic applications in farms. Results from the model simulations can be used to quantify the health risks associated with the discharge of residual veterinary antimicrobials to the environment.

Future work on the proposed mass load model can focus on incorporating the effects of in-soil degradation, farm practices such as manure storage and composting, and manure application methods. The residual environmental concentrations may also be estimated based on mass transport rather than on static mass balance methods alone. However, this approach may become too complex and tedious as data requirements expand rapidly. The advantage of a mass transport-based model is its ability to integrate farming practices (e.g. tilling), farm features (e.g., buffer strips), and other environmental processes (e.g., sediment transport, non-linear sorption) into the prediction calculations. It is therefore the task of the model user to find a balance between the complexity of using the model and the reliability of the model predictions. The proposed mass load model achieves this balance through its relatively minimal data requirements and its demonstrated accuracy in predicting the residual concentrations of priority antimicrobials.

Chapter 3: Occurrence and Distribution of Select Antimicrobials in the Grand River Watershed, Canada

This chapter discusses the findings of a field survey sampling conducted in the Grand River Watershed in Ontario, Canada in Fall 2013. It comprises five sections, namely: literature review, methodology, results, discussion, and summary. The chapter will be submitted as a research manuscript nearly in its entirety to Water Research, with co-authors SA Pagsuyoin (research supervision and manuscript review), L Bragg (development of method for chemical chromatographic analysis and supervision of laboratory analysis), and M Servos (research guidance and manuscript review).

3.1 Literature Review

The persistence of antimicrobials in the environment is a rising global concern due to its close link to antimicrobial resistance (AMR). Globally, antimicrobials, including medically important antibiotics, have been detected in various environmental media at concentrations ranging from parts per trillion to parts per billion (Kolpin et al., 2002). The majority of these measured values are below the 1 μg/L threshold recommended for a Phase II risk assessment by the Committee for Medicinal Products for Veterinary Use (CVMP) of the European Union. However, more studies are still needed to establish synergistic and chronic exposure effects in aquatic and terrestrial organisms (Kemper, 2008; Flaherty and Dodson, 2005).

Available literature on the environmental occurrence of antimicrobials mostly focuses on wastewater effluents and antimicrobials for human medication. For example, Guerra et al. (2014) examined the removal efficiencies of pharmaceuticals in six wastewater treatment plants and found that antimicrobials (antibiotics and antifungals) were more likely to sorb to biosolids. Miao et al. (2004) detected several human antimicrobials in treated effluents but only at concentrations below 1 μg/L. Human antimicrobials have also been detected in streams that receive wastewater effluents; for instance, medically important

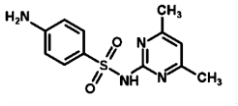
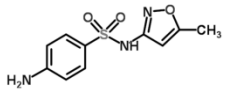
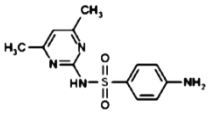
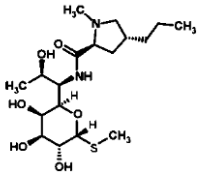
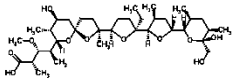
antimicrobials (e.g., tetracycline, trimethoprim, erythromycin) have been detected in rivers in North America (Forrest et al., 2011; Waiser et al., 2011; Verma et al., 2007; Kolpin et al., 2002).

In general, watershed-scale studies on the transport of antimicrobials are limited compared to similar studies for traditional pollutants such as nitrogen and phosphorus (Iglesias et al., 2014). The attenuation of a few antimicrobials (e.g., triclosan, triclocarban) in effluent-receiving streams has been studied (Arlos et al., 2014; Gautam et al., 2014; Singer et al., 2002). The transport of veterinary antimicrobials in small farms, shallow streams, and small watersheds has also been evaluated (Joy et al., 2013; Forrest et al., 2011; Lissemore et al., 2006). Recent research findings have highlighted the need to further examine the environmental occurrence of antimicrobials as well as the impact of runoff and effluent discharge on inducing AMR in organisms (Finley et al., 2013a; Kümmerer, 2009; Kemper, 2008). In Canada, despite the presence of large agricultural areas with high livestock production, there has not been any large-scale reconnaissance survey of antimicrobials in surface waters similar to that done by Kolpin et al. (2002) in the United States.

The widespread consumption and the environmental persistence of antimicrobials, along with the potential for inducing resistance traits in organisms, necessitate a more comprehensive understanding of antimicrobials fate and transport in the environment. With this in mind, the primary goal of the current study is to examine the patterns of occurrence of priority antimicrobials in a mixed-use watershed. Previous researchers have also underscored the need to identify the sources of residual antimicrobials in the environment to formulate appropriate strategies for managing health risks associated with AMR in pathogens (Finley et al., 2013a; Kümmerer, 2009). Thus, the second goal of this study is to provide insights on the point and non-point origin of frequently detected antimicrobials. Five priority antimicrobials from three classes were selected on the basis of their consumption in human and animal

medication: sulfamethazine, sulfamethoxazole, trimethoprim, lincomycin, and monensin (Table 3-1). Sulfamethazine and sulfamethoxazole belong to the sulphonamide class of antimicrobials. Sulphonamides competitively inhibit an enzyme (dihydropteroate synthetase) in microorganisms by reducing the uptake of p-aminobenzoic acid needed for dihydrofolic acid synthesis (Talwar and Srivastava, 2006; Grande et al., 2001). They are the second most prescribed antimicrobials worldwide (Kim et al., 2011) and are one of the top five most prescribed antimicrobials in Canada (CIPARS, 2012b). Sulfamethazine is one of the most commonly used veterinary antimicrobials from the sulphonamide class, while sulfamethoxazole is a human antimicrobial used to treat urinary tract infections, bronchitis, and sinusitis. Trimethoprim, a diaminopyrimidine, is prescribed for the treatment of infections of the respiratory and urinary tract in humans. Lincomycin is a type of lincosamide that is used to treat bacterial infections that cause dysentery, pneumonia and proliferative enteritis in swine (CFIA, 2014). Monensin, a member of the ionophore class, targets protozoa and bacteria. It is mainly used as a coccidiostat in poultry and as a growth promoter in cattle. It is also administered in low doses to increase feed efficiency in lactating dairy cattle (CFIA, 2011). Three chemicals, namely, ibuprofen, venlafaxine, and atrazine, were also included in the analyte list to indicate point and non-point sources. Ibuprofen and venlafaxine are used in human medication; ibuprofen is an anti-inflammatory drug while venlafaxine is an antidepressant. Atrazine is an herbicide used in the control of weeds and pests in crop farms, residential lawns, and golf courses (Sherchan and Bachoon, 2011).

Table 3-1: Properties of the Target Antimicrobials

Antimicrobial	Molecular Weight	Water Solubility (mg/L)	pKa	log K_{ow}	Structure
Sulfamethazine	278.3	1500[1]	2.07, 7.6[2]	0.19[1]	
Sulfamethoxazole	253.3	370[3]	1.85, 5.6[2]	0.89[4]	
Trimethoprim	290.3	400[1]	7.1[1]	0.91[1]	
Lincomycin	406.5	927[5]	7.64[6]	-0.25[6,]	
Monensin	670.9	0.003[1]	6.6[1]	5.43[1]	

1) Iglesias et al., 2014 2) Yu et al., 2011 (pKa 1 and pKa 2 respectively) 3) Tolls, 2001
4) Yargeau et al., 2007 5) Subedi et al., 2014 6) Kuchta et al., 2009

3.2 Methodology

3.2.1 Study Site

The study area is the Grand River Watershed (Figure 3-1), the largest watershed in Southern Ontario, Canada that drains an area of 6800 km^2. It is divided into 11 sub-basins: (1) Upper Grand, (2) Upper Middle Grand, (3) Middle Grand, (4) Lower Middle Grand, (5) Lower Grand, (6) Conestogo, (7) Fairchild Creek, (8) Mckenzie Creek, (9) Nith, (10) Speed and (11) Whiteman's Creek. Urban centres are concentrated in the Middle Grand and Speed basins. Livestock operations are heavily concentrated in the Upper Grand, Conestogo, and Nith basins. The watershed's river network consists of a 300-km main channel and three primary tributaries. Ninety-three percent of the land in the watershed is considered rural

(farmlands and forest). The watershed supports manufacturing and commercial industries, as well as intensive agriculture. It has approximately 6000 farms with a collective annual production of 290,000 cattle, 500,000 hogs, and 8.8 million poultry in addition to various crops (Farwell et al., 2008). There are also a total of 30 municipal wastewater treatment plants that serve a population of 985,000 (Chapman and Anderson, 2011). This population is projected to reach 1.4 million in 2031 partly due to the watershed's proximity to the City of Toronto and the Golden Horseshoe region (Farwell et al., 2008).

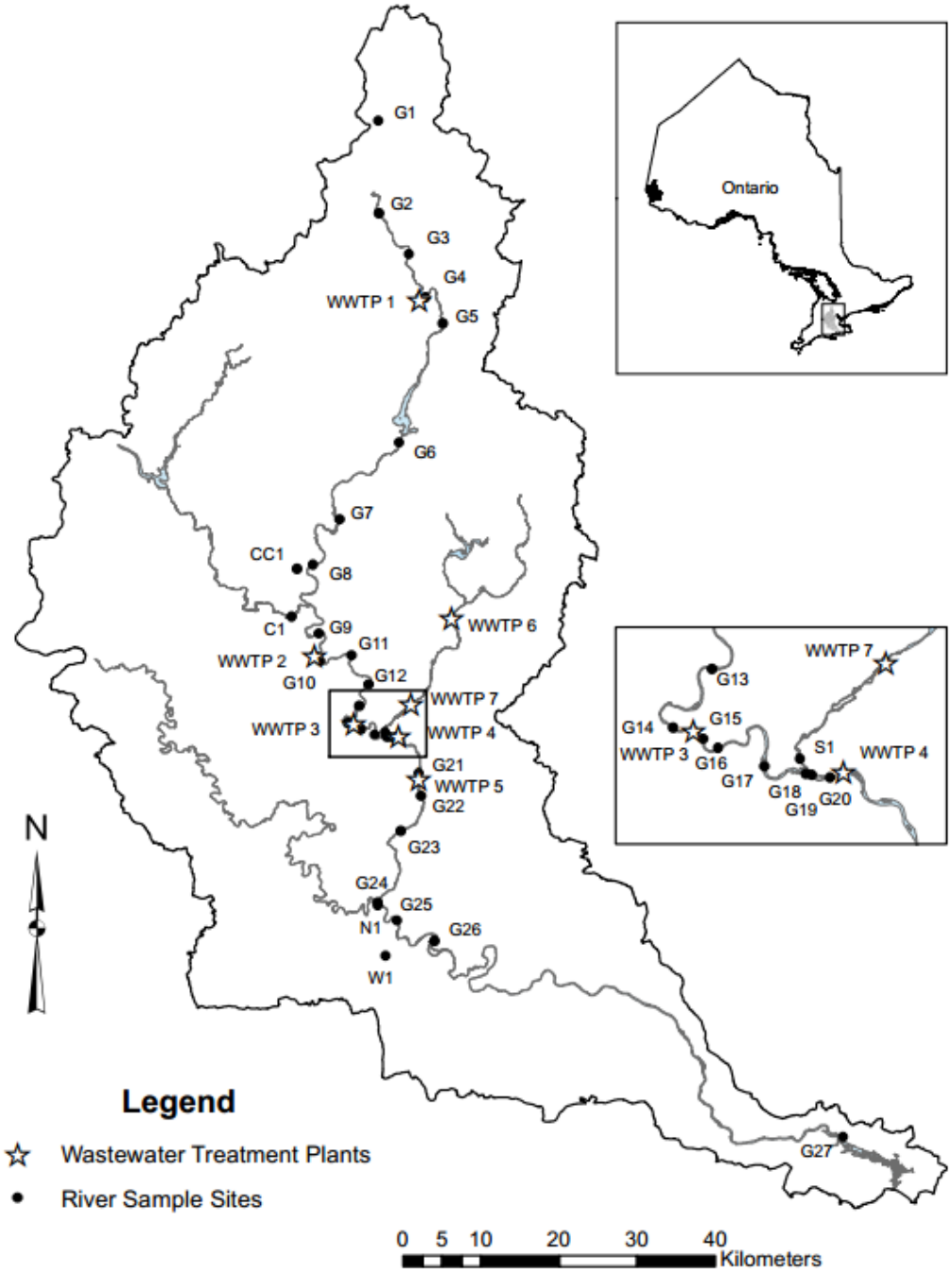


Figure 3-1: Map of the Grand River Watershed and Sampling Locations. G*i* labels indicate sampling sites in the main channel; CC1 indicates sampling site in the Canagagigue Creek in the Upper Middle Grand sub-basin; C1 indicates sampling site in Conestogo River; N1 indicates sampling site in the Nith River tributary; S1 indicates sampling site in the Speed tributary; W1 indicates sampling site in Whiteman's Creek; and WWTP*i* labels indicate effluent sampling sites.

3.2.2 Sample Collection

A total of 117 water samples were collected over a 2-day sampling event in early December 2013 from 32 locations (27 from the main channel and 5 from incoming creeks or tributaries) along the Grand River Watersheds' river network (surface water) and from 7 wastewater treatment plants (effluent) located within the watershed (5 from the main channel, 2 from the Speed River tributary) (Figure 3-2). Sampling sites were selected based on sub-basin characteristics (e.g., predominant land use) and on results from preliminary sampling efforts that indicated the presence of antimicrobials at these locations. Triplicate samples were taken at equidistant locations within each reach, one each from the left bank, right bank, and mid-channel. However, due to safety considerations, samples were taken only from one side of the river at three reaches (G10, 112 km downstream; G15, 133 km downstream; and G25, 176 km downstream). The river samples were collected in 500-mL amber glass bottles covered with Teflon®-lined screw caps and preserved on-site with 2.5 mL of 200 g/L sodium azide solution and 1.25 mL of 20 g/L ascorbic acid solution. The effluent samples were collected in 125-mL amber glass bottles and preserved similarly to the river samples. All bottles were filled to the brim to prevent analyte degradation, and kept in coolers maintained below 4°C until sample processing. Water temperature, pH, and conductivity were also measured onsite using a YSI ProfessionalPlus multimeter outfitted with a quarto-cable (YSI; Yellow Springs, OH). Additional water samples were collected in polyetheylene bottles for nutrient and chloride analyses (500 mL for nitrate, nitrite, total phosphorus and dissolved chloride analyses, and 250 mL for total ammonia). These were analyzed by an external laboratory (Maxxam Analytics, Waterloo, Ontario).

Temporal water sampling was also performed at four locations in the Nith River tributary and two in the Grand River (immediately above and below the confluence of the Nith and Grand Rivers). Sampling was

performed five times over an approximate two-week interval, from the end of September to early December 2013.

3.2.1 Materials Preparation and Storage

Chemical reagents, analyte standards (≥ 98% pure), and solvents (HPLC-grade) were purchased from Sigma Aldrich® (Oakville, Ontario). Deuterated standards (atrazine-d_5, ibuprofen-d_3, sulfamethazine-d_4, sulfamethoxazole-d_4, trimethoprim-d_3, and venlafaxine-d_6 HCl) were purchased from CDN Isotopes Inc. (Pointe-Claire, Quebec). Stock solutions of the analytes were prepared in methanol and stored in 8-mL amber glass vials at -20°C. Working solutions were prepared from these stock solutions just prior to chromatographic analyses.

3.2.2 Sample Extraction and Analysis

All samples were processed within 48 hours of collection. The raw water samples were filtered using a 0.3 μm x 47 mm glass fiber filter (Type A/E, Pall Life Sciences) to remove suspended particulate matter. Filtrates were adjusted to pH 2 with 10 N HCl, spiked with 100 μL deuterated standards, and loaded onto preconditioned Bond Elute Plexa cartridges (500 mg / 6cc, Agilent Technologies) at the rate of 5 mL/min. Solid phase extraction was performed using Dionex AutoTrace 280 (Thermo-Fisher Scientific) or a vacuum manifold (Visiprep, Supelco). A separate test was performed to confirm that there was no statistical difference between the recovery rates using either extraction equipment. The cartridges were preconditioned with 5 mL methanol and equilibrated with 5 mL LCMS-grade water. The loaded cartridges were eluted with 6 mL methanol. Eluates were vacuum dried to dryness under a gentle stream of nitrogen, reconstituted with 500 μL methanol, transferred to 2-mL HPLC vials, and stored at -20°C until chromatographic analysis.

Analyte concentrations were measured using an Agilent 1200 HPLC with an Applied Biosystems 3200 QTRAP® mass spectrometer equipped with an electrospray ionization source and a triple quadropole

analyzer. Analyte separation was achieved in an Agilent Eclipse® C18 column (4.6 mm × 150 mm × 5.0 μm). A mobile phase mixture of 5mM of ammonium acetate solution (A) and HPLC-grade methanol (B) was pumped through the column at a rate of 0.8 mL/min using a gradient program in both positive and negative modes held at 25 °C. Except for ibuprofen, all target compounds were detected in positive mode. Ibuprofen, along with other analytes not reported in this paper, was detected in negative mode. For the positive mode, the elution gradient was as follows: 0-0.5 min: 90% A, 0.5-8 min: 50→0% A, 8-10 min: 0% A, 10-15 min: 90% A. For the negative mode, the elution gradient was as follows: 0-0.5 min: 90% A, 0.5-8 min: 60→0% A, 8-10 min: 0% A, 10-15 min: 90% A. The sample injection volume was 20 μL and the block heater temperature was 25 ˚C. Nitrogen was used as the nebulizing and heating gas. Tracked precursor and product ions, as well as the collision energies for the analytes are listed in Table 3-2.

Table 3-2: Compound Dependent LC-MS/MS Parameters

Compound	Precursor (m/z)	Product (m/z)	Collision Energy (Volts)
Antimicrobials			
Sulfamethazine	279	92	41
Sulfamethoxazole	254	156	22
Trimethoprim	291	261	32
Lincomycin	407	126	50
Monensin	693	675	56
Chemical Indicators			
Ibuprofen	205	161	-11
Venlafaxine	278	58	42
Atrazine	216	174	27
Deuterated Compounds			
d-Sulfamethazine	283	96	43
d-Sulfamethoxazole	260	121	37
d-Trimethoprim	294	230	31
d-Ibuprofen	208	164	-10
d-Venlafaxine	284	64	35
d-Atrazine	22	179	22

3.2.3 Quality Control and Quantitation

All glassware were triple-rinsed in distilled de-ionized (DI) water and washed with methanol prior to use. The calibration curves for each analyte were constructed by preparing serial dilutions (0 to 500 μg/L) from the working solutions. Quantitation was performed using the Analyst® version 1.5.2 software (Applied Biosystems). During the chromatographic analysis, methanol blanks were injected between samples to prevent carry over.

3.2.4 Method Detection Limits and Recoveries

The method detection limits (MDLs) for the test compounds were assessed following the US EPA protocol (US EPA, 1997). Seven one-liter river water samples were each spiked with the test compounds to a final concentration of 10 ng/L (per compound) and extracted similarly to the field samples. For the method recoveries, seven one-liter river water samples were each spiked with the test compounds to a final concentration of 20 ng/L (per compound) and extracted similarly to the field samples.

3.3 Results

3.3.1 MDLs and Recoveries

Table 3-3 shows the method detection limits and recoveries for the test compounds. Detection limits in the river water ranged from 1.0 ng/L (sulfamethoxazole, lincomycin, and monensin) to 5.6 ng/L (sulfamethazine). Detection limits in the wastewater were assumed to be 5 times the detection limit in the river water since the volume of collected river samples was 5 times that of the effluent samples. Analyte recoveries from matrix-spiked samples were between 54-145%.

Table 3-3: Method Recoveries and Detection Limits (n = 7)

Compound	River Water MDL (ng/L)	Wastewater MDL (ng/L)	Recovery (%)
Antimicrobials			
Sulfamethazine	5.6	28	84-145
Sulfamethoxazole	1.0	5.0	54-91
Trimethoprim	1.2	6.0	71-91
Lincomycin	1.0	5.0	84-115
Monensin	1.0	5.0	60-140
Chemical Indicators			
Ibuprofen	2.2	11	67-145
Venlafaxine	1.4	7.0	70-130
Atrazine	1.2	6.0	62-92

3.3.2 Detection Frequencies and Detection Levels

Table 3-4 shows the detection frequencies, concentration ranges and mean concentrations of the target antimicrobials and chemical indicators in the river water samples and the wastewater effluent samples. In the river water samples, sulfamethoxazole had the highest detection frequency (84%) and mean concentration (36 ng/L), followed by trimethoprim (72%, with a mean concentration of 10 ng/L). Sulfamethazine was detected in 70% of the samples at a mean concentration of 16 ng/L, while lincomycin was detected at a higher concentration (21 ng/L) but only in a few samples (6.2%). Monensin was not detected in either the river water or effluent samples. For the chemical indicators, atrazine had the highest detection frequency (95%) and the lowest mean concentration (11 ng/L). Ibuprofen had the lowest detection frequency (64%) and the highest mean concentration (57 ng/L). The maximum antimicrobial concentrations measured in individual river water samples were 31 ng/L for sulfamethazine, 104 ng/L for sulfamethoxazole, and 29 ng/L for trimethoprim. For the chemical indicators, the highest measured concentration in individual samples was 146 ng/L for ibuprofen.

For the water samples from the tributaries, sulfamethoxazole was the most frequently detected antimicrobial (53%) but only at a very low mean concentration (4.7 ng/L). Trimethoprim was detected in fewer samples (20%) at an even lower mean concentration (2.6 ng/L). Sulfamethazine was detected in 40% of the samples at a much higher mean concentration (72 ng/L). Lincomycin was not detected in the water samples from the tributaries. For the chemical indicators, atrazine was detected in all samples (mean concentration of 8.5 ng/L) while ibuprofen was not detected. Venlafaxine was detected in only 20% of the samples (mean concentration of 12 ng/L). The highest measured analyte concentrations in tributary samples were 98 ± 8.8 ng/L for sulfamethazine and 17 ± 1.6 ng/L for atrazine.

For the effluent samples, sulfamethoxazole was detected in nearly all of the effluent samples (95%), followed by trimethoprim (57%) and to a lesser degree, by sulfamethazine (19%). Venlafaxine was detected in all samples while ibuprofen was detected in 76% of the samples and atrazine was not detected. It is interesting to note that except for sulfamethazine, the detected concentrations of the analytes in the effluents were significantly higher than their counterparts in the river samples. The mean concentrations of sulfamethoxazole and venlafaxine in the effluents was four times that of the main channel. In contrast, the mean sulfamethazine concentrations were highest in the tributaries (about twice of the effluent), and lowest in the main channel (about a third of the effluent).

Table 3-4: Detection Frequencies and Mean Concentrations of Target Analytes[a]

Compound	Main Channel Samples			Tributary Samples			Wastewater Samples		
	Detection Frequency	Range (ng/L)	Mean (ng/L)	Detection Frequency	Range (ng/L)	Mean (ng/L)	Detection Frequency	Range (ng/L)	Mean (ng/L)
Antimicrobials									
Sulfamethazine	70%	< MDL[b] – 31	16 ± 6.3	40%	< MDL – 108	72 ± 26	19%	< MDL – 55	43 ± 7.7
Sulfamethoxazole	84%	< MDL – 104	36 ± 22	53%	< MDL – 15	4.7 ± 3.5	95%	< MDL – 446	120 ± 119
Trimethoprim	72%	< MDL – 29	10 ± 5.6	20%	< MDL – 3.6	2.6 ± 0.7	57%	< MDL – 65	57 ± 3.3
Lincomycin	6.2%	< MDL – 35	21 ± 13	0%	< MDL	< MDL	0%	< MDL	< MDL
Monensin	0%	< MDL	< MDL	0%	< MDL	< MDL	0%	< MDL	< MDL
Chemical Indicators									
Ibuprofen	64%	< MDL – 146	57 ± 31	0%	< MDL	< MDL	76%	< MDL – 440	102 ± 145
Venlafaxine	86%	< MDL – 78	21 ± 18	20%	< MDL – 14	12 ± 1.6	100%	15 – 362	162 ± 107
Atrazine	95%	< MDL – 28	11 ± 4.8	100%	2 – 18	8.5 ± 4.9	0%	< MDL	< MDL

[a] Sample means were calculated only for data from samples where analytes were detected (above MDL), while detection frequencies and ranges were calculated using data from all samples.
[b] < MDL – below method detection limit.

3.3.3 Occurrence of Antimicrobials in the Watershed

Figure 3-2 shows the mean concentrations of the frequently detected antimicrobials in the water samples taken from the main channel. The confluences of the sampled tributaries with the main channel as well as the entry points of the wastewater effluents are also indicated in the inset figure.

In the main channel, the headwaters (locations G1-G4) are generally pristine with respect to antimicrobials, as indicated by their non-detection in the upstream sampling sites. The detection of sulfamethoxazole at a very low concentration (5.9 ± 2.8 ng/L) in location G2 (19 km downstream) may be due to the discharge from a very small (1.1 MLD) tertiary wastewater treatment plant located 29 km upstream (not sampled in this survey). The antimicrobial concentrations begin to rise in urban areas following discharges from wastewater treatment plants and downstream of several agricultural tributaries. The detected antimicrobial levels are below the 1 μg/L threshold (for water) for a Phase II risk assessment specified by the CVMP (EMEA, 2000). The veterinary antimicrobial, sulfamethazine, was detected more frequently downstream of the confluence of the main channel with the Conestogo River, which is the primary stream in the Conestogo sub-basin. The Conestogo sub-basin supports a relatively higher cattle and poultry population density compared to the rest of the watershed (see Table 2-3 in Chapter 2). There was also a notable increase in sulfamethazine concentrations past the Nith River, the main tributary in the Nith watershed, which also supports a high livestock population. Sulfamethoxazole and trimethoprim concentrations increased past the effluent discharge point (G4) of the first measured wastewater treatment plant (0.8 MLD), and even more after the second measured wastewater treatment plant (42 MLD, river sampling location G10) to further downstream of the main channel. The sulfamethoxazole concentrations increased more markedly than the trimethoprim concentrations, which fluctuated around the value detected just after the discharge point of the second treatment plant.

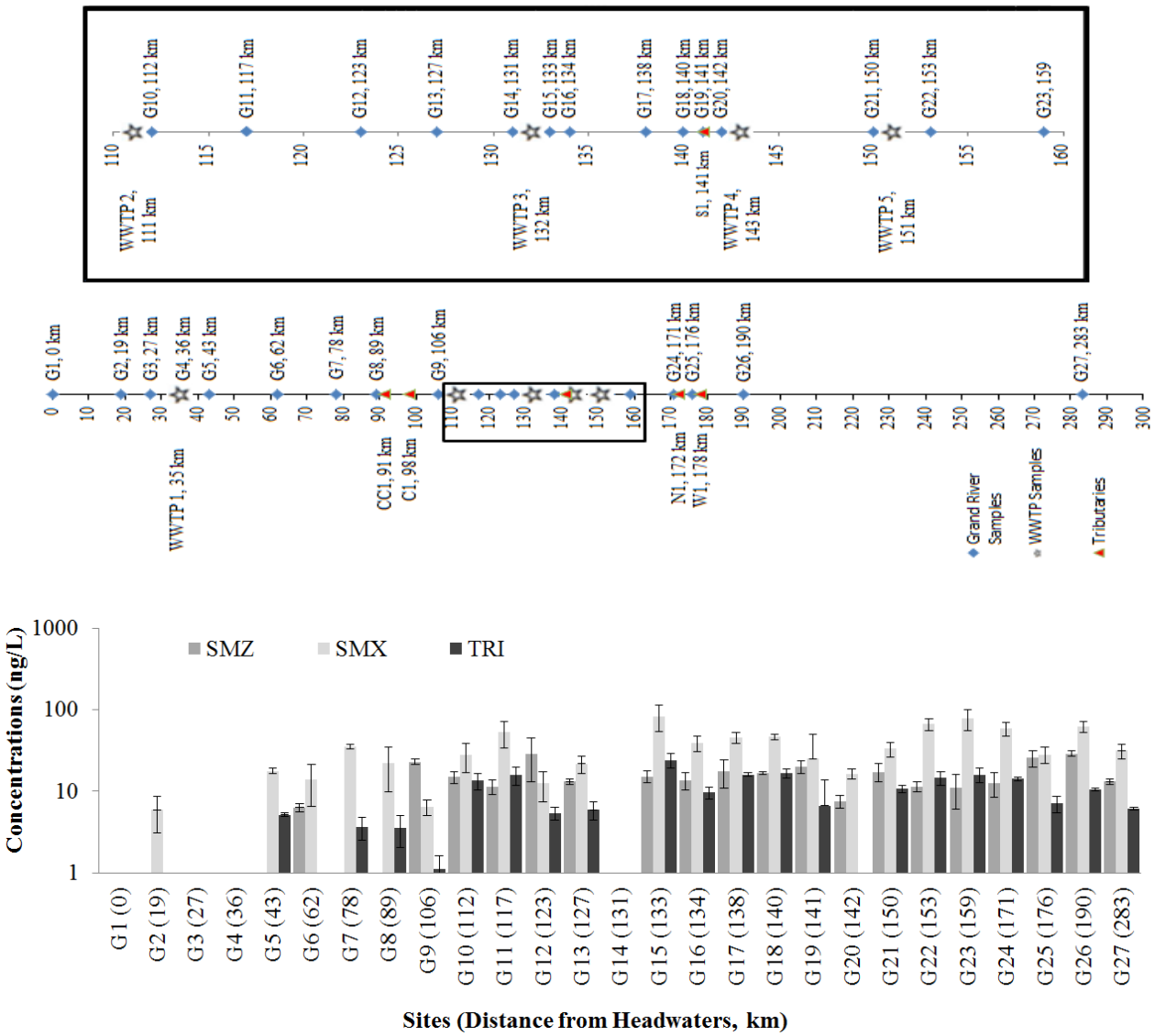


Figure 3-2: Concentrations of Antimicrobials in the Main Channel. Insert (top figure) Indicates Sampling Locations (dots) Confluence with Tributaries (triangles) and Entry points of Wastewater Effluent (stars). SMZ is Sulfamethazine, SMX is Sulfamethoxazole, and TRI is Trimethoprim. Error Bars Indicate ± Standard Deviation of Three Samples From Each Transect.

Figure 3-3 shows the measured concentrations of the antimicrobials in the five tributaries (Canagagigue Creek in the Upper Middle Grand sub-basin, Conestogo River in the Conestogo sub-basin, Speed River in the Speed sub-basin, Nith River in the Nith sub-basin, and Whiteman's Creek in the Whiteman's Creek sub-basin). Measured sulfamethazine concentrations were highest in the Conestogo and Nith Rivers. The corresponding sub-basins of these tributaries are largely agricultural, with the Nith sub-basin having the highest swine density among all sub-basins in the entire watershed (Table 2-3 in Chapter 2). Sulfamethoxazole was also measured in the other three tributaries (Conestogo River, Speed River, and Whiteman Creek) but only at very small concentrations compared to measurements from the main channel. A total of 13 municipal wastewater treatment plants, ranging in size from 0.13 to 54 MLD, discharge treated effluent to these tributaries (one in Canagagigue Creek, four in the Conestogo River, two in the Speed River, six in the Nith River, and none in Whiteman's Creek). Of these treatment plants, only effluents from the two treatment plants in the Speed River were sampled in this survey study.

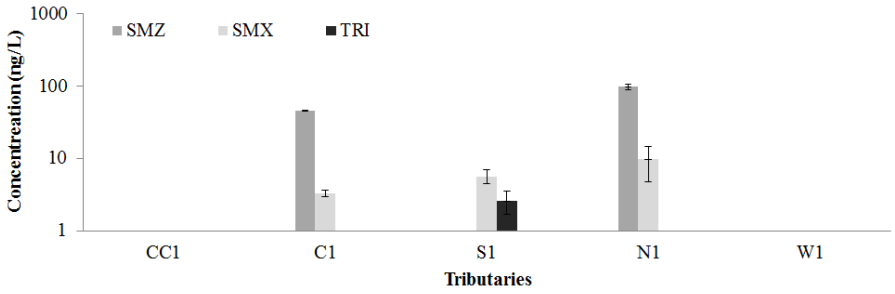


Figure 3-3: Concentrations of Antimicrobials in the Tributaries (CC1 is Canagagigue Creek, C1 is Conestogo River, S1 is Speed River, N1 is Nith River, and W1 is Whiteman Creek). SMZ is Sulfamethazine, SMX is Sulfamethoxazole, and TRI is Trimethoprim. Error Bars Indicate ± Standard Deviation of Three Samples From Each Transect.

3.3.4 Occurrence of Antimicrobials in the Wastewater Effluents

Figure 3-4 shows the antimicrobial concentrations in the effluents from seven wastewater treatment plants (WWTP1 to WWTP5 discharge to the main channel; WWTP6 and WWTP7 discharge to the Speed River).

The sulfamethazine concentrations in the effluents from WWTP1 to WWTP7 were small (all below MDL) except for two effluents (WWTP2, 46 ± 9.3 ng/L, in the main channel and WWTP7, 40 ± 4 ng/L in the Speed River tributary). Sulfamethoxazole and trimethoprim concentrations were higher in three treatment plants in the main channel (WWTP3-WWTP5) and in one treatment plant in the Speed River (WWTP7). The highest measured mean concentrations of sulfamethoxazole were 355 ± 126 ng/L and 199 ± 13 ng/L for WWTP4 and WWTP3, respectively, while for trimethoprim, the highest measured mean concentrations were 61 ± 3.7 ng/L and 58± 2.1 ng/L for WWTP3 and WWTP7, respectively.

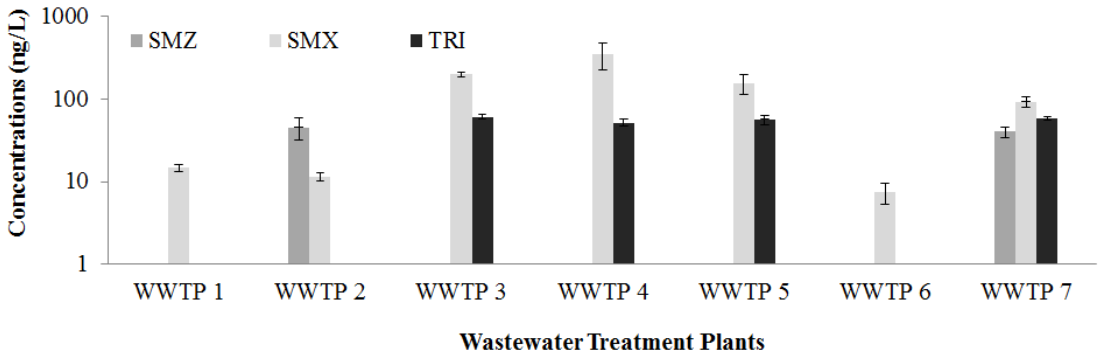


Figure 3-4: Concentrations of Antimicrobials in the Effluents. Discharge Points for WWTP1 to WWTP5 are Located Within the Main Channel While Discharge Points for WWTP6 and WWTP 7 are Within the Speed River. Whiteman Creek). SMZ is Sulfamethazine, SMX is Sulfamethoxazole, and TRI is Trimethoprim. Error Bars Indicate ± Standard Deviation of Three Replicate Samples.

3.4 Discussion

3.4.1 Detection of Antimicrobials in the Water

In this study, the antimicrobial concentrations measured in the effluent and river samples are several orders of magnitude lower than the 1 μg/L threshold set by the CVMP (10^1 to 10^3 lower). This threshold is based on a retrospective review of ecotoxicity data submitted in environmental assessments for public display; above this value, further risk assessment (Phase II) is recommended (EMEA, 2000; CDER, 1997). The

measured concentrations are also comparable to values that have been reported in previous studies (Table 3-5). The trimethoprim concentrations measured in the effluents are similar to the measured values for six wastewater treatment plants in the Lake Simcoe Watershed, a smaller watershed also located within the Southern Ontario region (Metcalfe, 2014). However, sulfamethoxazole concentrations are significantly higher (10^2 higher). The measured antimicrobial concentrations in this study differ from values previously reported by Lissemore et al. (2006) for the same watershed. Most notably, in this study, the veterinary antimicrobial monensin was not detected in the river samples while lincomycin was detected in very few samples (6%). In contrast, the previous study reported a higher detection frequency for these compounds (75% and 91%, respectively, for monensin and lincomycin). It should be noted that the study by Lissemore et al. involved temporal water sampling in only 8 locations while the current study involved one-time sampling in 32 river and tributary locations.

Table 3-5: Reported Antimicrobial Concentrations in River Water and Wastewater Effluent

Source	Location	n	Sulfamethazine (ng/L)	Sulfamethoxazole (ng/L)	Trimethoprim (ng/L)	Lincomycin (ng/L)	Monensin (ng/L)	Ref.
River	Zhujiang (China)	14	23	3.1	170	nm	nm	1
	Across USA	104	220	150	150	60	nm	2
	Across Germany	52	nd	30	nd	nm	nm	3
	Rio Grande (USA)	3	nm	300	nd	nd	nd	4
	Grand River Watershed (Canada)	125	3.2	2.8	2.7	12	44	5
	Grand River Watershed (Canada)	96	16	36	10	21	nd	6[a]
Wastewater Effluent	Guangzhou (China)	6	120	22	320	nm	nm	1
	Across Germany	10	nd	400	320	nm	nm	3
	Albuquerque (USA)	7	nm	310	180	nd	nd	4
	Across USA	12	nd	68	11	nd	nm	7
	Lake Simcoe Watershed (Canada)	7	nm	2.2	49	nm	nm	8
	Grand River Watershed (Canada)	21	42	120	57	nd	nd	6

nd = not detected nm = not measured a) Main channel only

1) Yang et al., 2011 2) Kolpin et al., 2002 3) Hirsch et al., 1999 4) Brown et al., 2006 5) Lissemore et al., 2006
6) This study 7) Glassmeyer et al., 2005 8) Metcalfe, 2014 (mean values calculated from reported data)

It is interesting to note that sulfamethazine, which is prescribed in Canada for use only in veterinary medicine (McEwen, 2002), was also detected frequently in wastewater effluents. This may be due to infiltration or contaminated overflows reaching the treatment plants. However, the detection of

sulfamethazine in treatment plant effluents is not rare. Sulfamethazine has also been detected in treated effluents in countries where this compound is used primarily in livestock (Guerra et al., 2014; Pérez et al., 2005).

3.4.2 Impact of Wastewater Treatment

The higher removal rates of pharmaceuticals, including antimicrobials, have been associated with greater degree of wastewater treatment, for example, advanced oxidation processes such as ozonation (Esplugas et al., 2007). In this study, lower effluent concentrations of the antimicrobials (sulfamethazine, sulfamethoxazole, trimethoprim) and chemical indicators (ibuprofen and venlafaxine) were measured from WWTPs with advanced treatment processes (Table 3-6). Sulfamethazine was not detected in the effluents of WWTP 1 and WWTP 6, which both have tertiary treatment. In contrast, sulfamethazine concentrations were higher in effluents from treatment plants that only have conventional activated sludge treatment (WWTP 2 and WWTP 7). Similarly, sulfamethoxazole and trimethoprim concentrations were also higher in the plants with secondary treatment systems. Effluent levels of ibuprofen and ammonia (see Appendix B) also follow a similar trend as the antimicrobials concentrations.

Table 3-6: Characteristics of Study Wastewater Treatment Plants

Plant	Treatment Level	Process	Receiving Water	Population Served	Average Flow (m^3/d)
WWTP 1	Tertiary	Extended Aeration	Grand River	2,726	800
WWTP 2	Secondary	2-Stage Conventional Activated Sludge	Grand River	129,502	42,000
WWTP 3	Secondary	Conventional Activated Sludge	Grand River	231,866	65,700
WWTP 4	Secondary	Conventional Activated Sludge	Grand River	20,699	8,400
WWTP 5	Secondary	Advanced Activated Sludge	Grand River	84,840	32,200
WWTP 6	Tertiary	Rotating Biological Contactors	Speed River	134,894	46,000
WWTP 7	Secondary	Modified High Rate Activated Sludge[a]	Speed River	24,824	6,600

Data from the Region of Waterloo Wastewater Treatment Master Plan Final Report (2007)
[a] WWTP 7 is an extended aeration plant but is operated as a conventional activated sludge process

3.4.3 Agriculture and Wastewater Inputs in the Receiving Water

Over-all, there was an increasing trend in antimicrobial concentrations in the Grand River from the headwaters to near its discharge point to Lake Erie (Figure 3-2). The more notable increases occurred beginning in the central region of the watershed (starting at location G7 in Figure 3-1). The concentrations of the veterinary antimicrobial sulfamethazine spiked downstream of the channel's confluences with agriculture-impacted tributaries (Conestogo and Nith Rivers). Although there are wastewater treatment plants upstream of the sampling locations in these tributaries, these have small capacities (0.1 – 2.7 MLD) and are unlikely the main sources of the sulfamethazine in the receiving stream. Effluents from these treatment plants were not sampled in the current study. However, data from the sampled WWTP effluents indicate that sulfamethazine concentrations are generally much lower than either sulfamethoxazole or trimethoprim concentrations (Table 3-4). Therefore, spikes in sulfamethazine concentration in the river are likely due to agricultural or other non-point sources.

On the other hand, the spikes in sulfamethoxazole and trimethoprim concentrations in the main channel occur just downstream of effluent discharge points. The concentrations of these compounds in the effluents of the treatment plants located along the main channel were also relatively high. Taking into account the capacities of the treatment plants, these data suggest that the sulfamethoxazole and trimethoprim in the main channel likely come from point sources.

In the main channel, the levels of ibuprofen, venlafaxine, nutrients, and chloride also exhibited notable increases just downstream of effluent discharges (Appendix B). Based on the higher concentrations of these chemicals in the effluents, it can be inferred that the WWTPs are the main sources of these chemicals in the main channel.

3.4.4 Correlations among Antimicrobials, Chemical Indicators, Chloride, and Nitrogen

A multivariate analysis of the mean concentrations (antimicrobials, chemical indicators, nutrients, and chloride) was performed to determine correlations among the detected chemicals in the river and effluent samples (Appendix D). Correlation coefficients (R^2 with $p < 0.001$) were calculated using the Data Analysis toolkit in Microsoft Excel®.

In river samples, the human antimicrobials sulfamethoxazole and trimethoprim were well correlated with each other and with venlafaxine ($R^2 > 0.7$), suggesting that venlafaxine may be a good indicator for these antimicrobials (co-occurrence in river samples). Sulfamethazine was poorly correlated with other measured compounds (R^2 of 0.7 – 0.33). Nutrients were also correlated to chemical indicators, some antimicrobials, as well as to other nutrients in river samples. Total ammonia was correlated to ibuprofen ($R^2 = 0.87$) and nitrite was correlated to trimethoprim ($R^2 = 0.75$). Dissolved chloride was correlated to nitrite and nitrate at $R^2 = 0.74$ and $R^2 = 0.72$, respectively. In the wastewater, sulfamethoxazole and

trimethoprim were highly correlated (R^2 = 0.74) probably because they are often prescribed together (Grande et al., 2001). Venlafaxine was also correlated to sulfamethoxazole and trimethoprim at R^2 = 0.82 and R^2 = 0.72, respectively. Total ammonia was correlated only to sulfamethazine (R^2 = 0.92). As seen in Figure 3-4, sulfamethazine was detected only in the WWTPs with poorer treatment, which is a similar trend observed for total ammonia.

The antimicrobials were poorly correlated with atrazine. As an herbicide, atrazine may be a good indicator of non-point source pollution due to its use for weed control in lawns. However, in this study, atrazine was not a suitable indicator for non-point source antimicrobials from farms. While atrazine was not detected in treatment plant effluents where neither lincomycin nor monensin was detected, it was also measured throughout the watershed where neither antimicrobial was found. These results indicate that atrazine may be a suitable marker for select contaminants from nonpoint sources (e.g., agriculture and land development) but not necessarily for the veterinary antimicrobials investigated in this study. The widespread low atrazine concentrations of atrazine in the river may be due to the seasonal application of atrazine, usually in spring, in non-agricultural areas such as golf courses (Winter and Dillon, 2005).

3.4.5 Temporal Variabilities

The results of the biweekly sampling in the Nith River and near its confluence with the main channel show variability from the results obtained during the one-time watershed-scale spatial sampling (Appendix C). Sulfamethazine was detected at lower concentrations (9 - 65 ng/L) during the biweekly sampling compared to the large-scale sampling (98 ± 8.8 ng/L). This variability highlights the role of timing during field sample collection for monitoring purposes. The large-scale spatial sampling in this study was conducted late in the fall season to coincide with the period when farmers typically spread manure fertilizer in the crop farms. The two sampling days also occurred after a rain event the previous days when antimicrobials

may have leached from soils and were rapidly flushed into the rivers. Except for a single trimethoprim measurement just slightly above the MDL (1.4 ng/L), sulfamethoxazole and trimethoprim were generally not detected during the biweekly sampling in the Nith River tributary. However, both were detected in the Grand River (confluence locations) but only at very low concentrations (sulfamethoxazole, 4.2 ± 2.8 ng/L; trimethoprim 5.8 ± 2.7 ng/L).

3.4.6 Implications for Water Resources Management

The data trends from this study demonstrate that both point and non-point sources influence the concentration profiles of antimicrobials in the Grand River Watershed. While the watershed is largely agricultural, the contribution of wastewater discharges to antimicrobial loads is also comparatively significant. This finding is consistent with other studies that indicate significant contributions of antimicrobials from point sources in largely agricultural watersheds (Iglesias et al., 2014; Brown et al., 2006; Hirsch, 1999). From the perspective of AMR risk assessment, this finding implies that contributions of antimicrobials from point and non-point sources should be treated with equal importance despite clear indications that agriculture is the primary land use within the watershed. The results of the temporal sampling also highlight the significance of accounting for seasonal changes in antimicrobial loads. Antimicrobials inputs from farms may appear as pulse inputs especially right after rain events and during the land tilling seasons. However, inputs from wastewater discharges tend to be generally constant throughout the year, although minor fluctuations may be expected as a result of fluctuations in treatment efficiency during the cold and warm seasons.

3.5 Chapter Summary and Future Work

This study provides empirical data on the occurrence of antimicrobials in a mixed-use watershed. Research findings provide useful information and insights for assessing the risks associated with the high consumption of antimicrobials in human medication and in the livestock industry. The measured

antimicrobial concentrations were comparable to findings from other studies of similar watersheds and treatment plant effluents. All measurements were below the 1 μg/L threshold for a Phase II risk assessment set by the CVMP. Antimicrobials concentrations increased downstream of treatment plant outfalls and discharges from agricultural tributaries. Wastewater effluents were the primary sources of human antimicrobials (sulfamethoxazole and trimethoprim) in the main channel, while agricultural tributaries were the primary sources of the veterinary antimicrobial, sulfamethazine.

Further research should explore the temporal variabilities in the antimicrobial levels in the watershed. While it can be expected that there are typically greater temporal fluctuations in non-point source inputs of veterinary antimicrobials compared to inputs of point-source human antimicrobials, these trends might not be observed in the Grand River in the coming years. It is anticipated that new and tighter regulations on the use of veterinary antimicrobials will soon take effect in Canada. Also, several wastewater treatment plants in the Grand River Watershed either have been recently upgraded or will be upgraded in the next three years. The findings of this study can serve as baseline for determining the effects of these new legislations and infrastructure upgrades on the occurrence of antimicrobials in the watershed.

Chapter 4: Conclusion and Future Work

In Chapter 1 of this manuscript, a comprehensive literature review was performed to identify some of the knowledge gaps pertaining to the global use, environmental occurrence, and regulations on the consumption and distribution of antimicrobials. The potential for developing resistant traits in pathogens is the most significant health risk associated with the widespread use of antimicrobials and their persistence in the environment. Quantifying this risk can assist policy makers in formulating corresponding mitigation strategies, however, the risk analyst faces the challenge of varying regulations among countries and the lack of available data on antimicrobials consumption. The current study contributes knowledge to this research gap by providing preliminary data on the occurrence and distribution of select antimicrobials in the environment through modeling and empirical data collection. The study addresses the following specific objectives:

(i) development of a mass load estimation model for determining the residual levels of veterinary antimicrobials in the environment, discussed in Chapter 2; and

(ii) analysis of the patterns of occurrence of relevant antimicrobials in a mixed use watershed, discussed in Chapter 3.

The mass load estimation model (Equation 1) presented in Chapter 2 has been verified with literature from and was applied to a case study of four veterinary antimicrobials (lincomycin, monensin, oxytetracycline, and sulfamethazine) in the Grand River Watershed in Ontario, Canada. Results of the model application suggest that the estimated residual concentrations of each target compound do not exceed the toxicity threshold for soil (100 μg/kg) for a Phase II risk assessment as recommended by the Committee for Medicinal Products for Veterinary Use (CVMP). The proposed estimation model can be used as a screening tool to assess the risks associated with the widespread use of antimicrobials in livestock farms,

and the potential effects of applying livestock manure to soil. It can also complement mass transport models in the study of the environmental attenuation of residual antimicrobials.

In Chapter 3, the occurrence of antimicrobials in the Grand River Watershed in Southern Ontario, Canada was studied by collecting a total of 117 water samples from 7 wastewater treatment plants and 32 sampling locations in the main river channel and in five tributaries. The samples were analyzed for five priority antimicrobials (human and veterinary), namely: lincomycin, monensin, sulfamethazine, sulfamethoxazole, and trimethoprim. The concentrations of antimicrobials in the main river channel generally increased downstream of wastewater treatment plants. Concentrations of the veterinary antimicrobial sulfamethazine also increased in the vicinity downstream of agricultural areas. The findings from this reconnaissance survey indicate that the antimicrobial concentrations in the water are below the 1 μg/L threshold set by the CVMP for a Phase II risk assessment. These findings also indicate that predominantly agricultural watersheds are a source of veterinary antimicrobials in the main river channel. Results of the biweekly water sampling in the Nith River highlight the temporal variability in the concentrations of antimicrobials, suggesting that temporal sampling is necessary in monitoring the environmental fate of residual antimicrobials.

In Canada, it is anticipated that new and tighter regulations on the use of veterinary antimicrobials will take effect in the coming years. Also, several wastewater treatment plants in the watershed either have been recently upgraded or will be upgraded in the next three years. The findings of this study can serve as baseline for determining the effects of these regulatory changes and infrastructure improvements on the spread of antimicrobials in the Grand River Watershed.

Future related work can be explored in the following areas:

(i) Improvements in the mass load model. Degradation as a result of manure storage, composting, or in-soil biochemical degradation can be accounted for in the proposed mass load estimation model presented in Chapter 2 by incorporating kinetics terms, for example, first-order or pseudo-first order degradation. A more robust method for estimating livestock densities can also be performed by accounting for variabilities in individual farm sizes where such data are available. However, these improvements should be weighed against the simplicity and ease of use of the current model;

(ii) Regular spatial and temporal monitoring of antimicrobial concentrations in the main river channel and in the agricultural tributaries. As previously noted, the data and findings from the current study can be used to compare the effects of future legislations and wastewater treatment plant upgrades, and regular monitoring will establish if any environmental improvements are observed over time; and

(iii) Sampling for antimicrobials in other environmental matrices: sludge, livestock manure, soil, and river sediments. Some antimicrobials (e.g., tetracycline) have a higher affinity for solids than the aqueous phase. In combination with environmental persistence, this chemical property may indicate that the soil matrix can act as a reservoir for antimicrobials and consequently, antimicrobial resistance.

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Appendix A: Validation Methodology

This appendix serves as a guide to provide further understanding as to how the model is used to estimate veterinary antimicrobial concentrations. The discussion provided in this appendix focuses on variable acquisition and the arguments for variable use within the validations and case study presented in Chapter 2 of this thesis.

The validations were performed using sulfamethazine and oxytetracycline data for swine and poultry from Wang et al., (2014) and sulfamethazine concentrations for swine from Zhou et al., (2013). This section discusses how values were chosen for variables in Equation 1 for the sulfamethazine calculation from Wang et al., (2014). The Zhou et al., (2013) and CVMP comparison portions of the validation was calculated in a similar manner.

$$\mathrm{PEC_s} = \left(\frac{\mathrm{D\ x\ T\ x\ B\ x\ L\ x\ F_h\ x\ } F_e}{\rho_s \mathrm{\ x\ d}}\, x\, \frac{A_p}{A_f}\right) \mathrm{C} \qquad \text{Equation 1}$$

Antimicrobial dosage (*D*) is the amount of sulfamethazine measured in feed (4.58 μg/kg) multiplied by the mass of feed consumed by a swine per day (2.5 kg/day). These values were used as presented by Wang et al., (2014). The number of treatment days (*T*) was found to be the number of days that it takes the herd to produce the manure spread with the smallest value being one (Equation 8). *T* was taken to be one for swine and two for poultry (Table A-1). The mass of manure spread was divided by the multiplication of three terms: the amount of manure produced by the livestock species, the rate of manure production and percent dry matter. In this validation, 25,000 swine excreted 6625 kg of manure in one day at a rate of 2 kg/day and 23% dry mass (Wang et al., 2014). A livestock density

(L) of 25,000 swine were used to calculate PEC_s. The body weight of the swine was assumed to be 106 kg, the average of the three hog groups in the CVMP (EMEA, 2008). If greater refinement is desired one can calculate an estimate from all three hog age groups and sum the estimates to get a refined estimate total for PEC_s.

The fraction of manure spread per area (F_a) takes into account whether all of the manure is spread from the livestock pens onto a field. The units for this term are $hectares_{farm\ pens}$ divided by $hectares_{field}$. If the density of the livestock is already in livestock/$hectares_{field}$, the F_a term can be one, as it is in Validation 1. The fraction of the herd being treated (F_h) was one as the paper makes no mention of different feeds for different hog growth stages. Similarly, the fraction excreted (F_e), the amount of sulfamethazine excreted by swine of the original compound, is also one despite being metabolized in the liver of swine. This is because sulfamethazine components inactivated in swine's liver by attached sugars can be reactivated by environmental bacteria when the sugars are consumed (Sarmah et al., 2006). Thus, sulfamethazine metabolites cannot be discounted following excretion. Bulk soil density (ρ_s), is 1150 kg m^3 (Wang et al., 2014). The depth of top soil (d) that manure infiltrated was 0.10 m, an average of the 0-0.2m of topsoil sampled (Wang et al., 2014). The conversion factor (C) was 1 hectare per 10,000 m^2. The PEC_s estimate for the Wang et al., (2014) hog farm is 26.38 µg/kg compared to the Wang et al., (2014) topsoil measurement of 18.89 ± 6.40 µg/kg. The difference between the estimate and measured values is 7.5 µg/kg or 140%.

Table A-1: Percentage of Canadian Herds Treated with Antimicrobial and Excretion Rates of Antimicrobial by Livestock Type

Antimicrobial	Poultry % herds treated[a]	% excreted	Cattle % herds treated	% excreted	Swine % herds treated	% excreted
Lincomycin	N/A	83[b]	10[e]	-	42[h]	21[b]
Monensin	N/A	94[c]	76[e]	95[f]	-	-
Oxytetracycline	N/A	100[d]	10[e]	100[d]	15[h]	100[d]
Sulfamethazine	N/A	-	5[e]	26[g]	8[h]	52[i]

*Dash indicates not extensively used in animal with no data available.

a. No herd use data available, Agunos et al., 2013 b. Hornish et al., 1987 c. Donoho et al., 1982
d. CVMP, 1995 e. Carson et al., 2008 f. Herberg et al., 1978. g. Nouws, 1992.
h. CIPARS, 2008 i. Paulson et al., 1981.

Table A-2: Livestock Specific Weights and Average Livestock Weights Used in the Model

Livestock	Individual Animal Body Weight (kg)[1]	Average Body Weight (kg)
Cattle		295
Calf	140	
Cow	450	
Swine		106
Weaner	12.5	
Fattening	65	
Sow (with litter)	240	
Chickens		1.3
Broiler	1.0	
Layer	1.6	

[1]Individual Body Weights taken from EMEA, 2008.

Case Study

The choices for three variables applied in the case study that differed from the validation variable acquisition method will now be discussed. These variables include the livestock density, soil bulk density, and fraction of herd treated.

The livestock density (L) in the case study was calculated as the summed county populations within a sub-basin area. Livestock populations were assumed to be equally distributed across the county[1]. The boundary of the Grand River Watershed and Ontario counties were overlaid to determine the percentage of county area within a sub-basin[2]. The area of the county within the watershed (taken as a percent) was multiplied by the county livestock population to obtain the number of livestock in the sub-basin contributed by that county. The fragmented livestock county populations were then summed within the sub-basin boundaries to form each sub-basin livestock population. This method of averaging livestock populations has been performed elsewhere in the research literature (Dorner et al., 2006).

The use of only one dry soil bulk densities were used due to their dominance in the top soil of most agricultural catchments (GRCA, 2008). The dry soil bulk density of Guelph loam (1550 kg m^{-3}) soil was used (GRCA, 2008; Reynolds et al., 2002). The depth of manure penetration is the distance that the manure travels into the soil. This does not take incorporation or injection of manure into account but assumes surface level application with solid or liquid manure. Depth of manure penetration was assumed to be 0.10 m which is consistent with past studies (EMEA, 2008). A unit conversion factor is present in Equation 1 (C) and is equal to 0.1 in this case study. It is comprised of a factor of 1000 μg /mg in the numerator divided by 10,000 m^2/ha in the denominator.

The fraction of livestock herds treated with the select antimicrobial in Canada (F_h) was taken from CIPARS, (2008) for swine and Carson et al., (2008) for cattle for the case study. Currently there is no herd data available for poultry antimicrobial use due to a lack of national surveillance (Agunos et al., 2013). Therefore, an assumption was made the antimicrobial is administered to all poultry herds.

[1] Livestock county population data was obtained from the Ontario Ministry of Agriculture and Food (OMAFRA) website titled "2011 Livestock by County and District at a Glance" with original data generated by Statistics Canada.

[2] Watershed and county ArcGIS® layers were provided by the University of Waterloo GeoSpatial Centre.

Appendix B: Antimicrobial Concentrations for Grand River

			Compound Concentrations (ng/L)															
Site			Sulfamethazine		Sulfamethoxazole		Trimethoprim		Lincomycin		Monensin		Venlafaxine		Ibuprofen		Atrazine	
Grand River	Latitude	Longitude	Mean	Std. Dev	Mean	Std. Dev	Mean	Std. Dev	Mean	Std. Dev	Mean	Std. Dev	Mean	Std. Dev	Mean	Std. Dev	Mean	Std. Dev
G1	44.096377	-80.3735	< MDL	-	< MDL	-	< MDL	-	< MDL	-	< MDL	-	4.2	0.6	< MDL	-	< MDL	-
G2	43.990018	-80.374	< MDL	-	5.9	2.8	< MDL	-	< MDL	-	< MDL	-	< MDL	-	22	2.9	1.9	0.2
G3	43.942391	-80.3265	< MDL	-	< MDL	-	< MDL	-	< MDL	-	< MDL	-	1.6	0.1	< MDL	-	5.8	3.1
G4	43.892852	-80.3003	< MDL	-	< MDL	-	< MDL	-	< MDL	-	< MDL	-	5.1	3.2	< MDL	-	4.0	0.6
G5	43.862559	-80.273	< MDL	-	18	1.4	5.1	0.3	< MDL	-	< MDL	-	11	0.9	< MDL	-	11	0.3
G6	43.725181	-80.3445	6.3	0.7	14	7.5	< MDL	-	< MDL	-	< MDL	-	53	33	17	3.9	4.6	1.2
G7	43.637135	-80.4406	< MDL	-	35	2.7	3.7	1.2	< MDL	-	< MDL	-	10	0.5	< MDL	-	13	2.9
G8	43.585202	-80.4824	< MDL	-	22	12	3.5	1.5	< MDL	-	< MDL	-	10	1.7	< MDL	-	7.7	1.4
G9	43.505204	-80.4745	23	1.6	6.4	1.3	1.7	-	< MDL	-	< MDL	-	< MDL	-	42	-	19	8.8
G10	43.473609	-80.473	15	2.5	28	11	14	3.1	< MDL	-	< MDL	-	18	4.5	146	67	14	0.7
G11	43.480235	-80.423	11	2.4	53	19	16	4.1	< MDL	-	< MDL	-	54	7.1	99	36	13	2.2
G12	43.446298	-80.3959	29	16	12	5.1	5.3	1.0	2.0	-	< MDL	-	5.6	0.2	81	18	11	3.8
G13	43.421811	-80.4113	13	0.9	22	5.2	5.9	1.4	< MDL	-	< MDL	-	17	0.6	65	18	23	5.6
G14	43.401992	-80.4296	< MDL	-	< MDL	-	< MDL	-	< MDL	-	< MDL	-	< MDL	-	< MDL	-	9.3	0.8
G15	43.398063	-80.4156	15	2.4	83	30	24	4.8	< MDL	-	< MDL	-	56	4.8	47	6.4	10	1.7
G16	43.394999	-80.4085	14	3.2	39	8.0	10	1.7	< MDL	-	< MDL	-	10	3.0	68	7.8	8.9	2.6
G17	43.388404	-80.387	18	6.6	45	6.9	16	0.9	< MDL	-	< MDL	-	17	1.1	80	21	13	3.3
G18	43.385963	-80.3676	17	0.6	47	3.7	17	2.2	< MDL	-	< MDL	-	14	1.2	85	18	14	1.9
G19	43.385488	-80.3648	20	3.7	25	25	6.6	7.0	35	-	< MDL	-	13	3.8	55	38	12	4.6
G20	43.384609	-80.3564	8	1.3	16	2.2	< MDL	-	< MDL	-	< MDL	-	1.9	0.1	16	-	11	0.7
G21	43.343442	-80.3171	17	4.3	33	6.7	11	1.1	< MDL	-	< MDL	-	10	0.4	59	7.2	10	1.8
G22	43.317468	-80.3147	11	1.6	66	11	15	2.6	< MDL	-	< MDL	-	41	0.9	40	22	9.4	1.1
G23	43.277158	-80.347	11	5.0	78	23	16	3.2	< MDL	-	< MDL	-	47	6.1	44	7.0	9.2	2.7
G24	43.19511	-80.384	13	4.3	59	11	14	0.8	< MDL	-	< MDL	-	48	2.7	33	-	18	7.0
G25	43.173667	-80.3534	26	5.8	28	6.4	7.1	1.6	< MDL	-	< MDL	-	12	0.3	48	8.7	14	3.8
G26	43.149842	-80.2948	29	2.1	62	10	11	0.4	< MDL	-	< MDL	-	44	2.1	28	4.5	10	0.3
G27	42.917841	-79.6555	13	1.1	32	6.4	6.1	0.3	22	13	< MDL	-	11	0.8	63	33	20	10

			Compound Concentrations (ng/L)															
Site			**Sulfamethazine**		**Sul[illegible]ethoxazole**		**Trimethoprim**		**Lincomycin**		**Monensin**		**Venlafaxine**		**Ibuprofen**		**Atrazine**	
Tributaries	**Latitude**	**Longitude**	**Mean**	**Std. Dev**	**Mean**	**Std. Dev**	**Mean**	**Std. Dev**	**Mean**	**Std. Dev**	**Mean**	**Std. Dev**	**Mean**	**Std. Dev**	**Mean**	**Std. Dev**	**Mean**	**Std. Dev**
CC1	43.58069	-80.5081	< MDL	-	< MDL	-	< MDL	-	< MDL	-	< MDL	-	< MDL	-	< MDL	-	2.0	0.5
C1	43.52546	-80.5167	46	1.1	3.3	0.4	< MDL	-	< MDL	-	< MDL	-	< MDL	-	< MDL	-	17	1.6
S1	43.391	-80.370249	< MDL	-	5.7	1.2	2.6	0.9	< MDL	-	< MDL	-	12	2.0	< MDL	-	6.5	1.1
N1	43.19148	-80.3837	98	8.8	9.7	4.9	< MDL	-	< MDL	-	< MDL	-	< MDL	-	< MDL	-	11	1.6
W1	43.13309	-80.3724	< MDL	-	< MDL	-	< MDL	-	< MDL	-	< MDL	-	< MDL	-	< MDL	-	6.6	1.8
WWTPs																		
WWTP1	43.889403	-80.3108	< MDL	-	15	1.4	< MDL	-	< MDL	-	< MDL	-	110	0.7	15	-	< MDL	-
WWTP2	43.479573	-80.4822	46	13	12	1.2	< MDL	-	< MDL	-	< MDL	-	17	2.0	52	3.7	< MDL	-
WWTP3	43.400846	-80.4202	< MDL	-	199	13	61	3.7	< MDL	-	< MDL	-	119	7.5	37	8.6	< MDL	-
WWTP4	43.386408	-80.3501	< MDL	-	355	126	52	4.8	< MDL	-	< MDL	-	349	11	53	6.0	< MDL	-
WWTP5	43.338312	-80.3179	< MDL	-	156	41	57	7.4	< MDL	-	< MDL	-	240	30	30	2.4	< MDL	-
WWTP6	43.52177	-80.264162	< MDL	-	7.5	2.2	< MDL	-	< MDL	-	< MDL	-	69	3.0	< MDL	-	< MDL	-
WWTP7	43.42389	-80.329962	40	6	93	13	58	2.1	< MDL	-	< MDL	-	228	3.2	426	24	< MDL	-

Appendix C: Antimicrobial Concentrations for Nith Sub-basin Sampling

Antimicrobials

Sulfamethazine Temporal Sampling										
	Sept. 30		Oct. 21		Nov. 6		Nov. 19		Dec. 6	
Site	Mean	Std. Dev	Mean	Std. Dev	Mean	Std. Dev	Mean	Std. Dev	Mean	Std. Dev
A	< MDL	-	17	3.0	13	4.1	27	5.4	48	21
B	< MDL	-	28	3.0	25	7.3	31	6.1	< MDL	-
C	< MDL	-	15	2.8	25	-	26	2.1	< MDL	-
D	< MDL	-	17	9.4	29	3.4	< MDL	-	< MDL	-
G24	< MDL	-	16	2.2	11	1.1	65	27.5	8.8	-
G25	< MDL	-	< MDL	-	10	1.5	48	6.4	9.2	0.8

< MDL indicates less than method detection limit.
Dash indicates no standard deviation available (n < 2).

Sulfamethoxazole Temporal Sampling										
	Sept. 30		Oct. 21		Nov. 6		Nov. 19		Dec. 6	
Site	Mean	Std. Dev	Mean	Std. Dev	Mean	Std. Dev	Mean	Std. Dev	Mean	Std. Dev
A	< MDL	-	< MDL	-	< MDL	-	< MDL	-	< MDL	-
B	< MDL	-	< MDL	-	< MDL	-	< MDL	-	< MDL	-
C	< MDL	-	< MDL	-	< MDL	-	< MDL	-	< MDL	-
D	< MDL	-	< MDL	-	< MDL	-	< MDL	-	< MDL	-
G24	< MDL	-	5.0	1.4	2.1	0.3	1.4	0.1	8.6	1.2
G25	< MDL	-	4.8	0.9	1.9	0.4	1.8	0.5	7.6	0.7

< MDL indicates less than method detection limit.
Dash indicates no standard deviation available (n < 2).

Trimethoprim Temporal Sampling										
	Sept. 30		Oct. 21		Nov. 6		Nov. 19		Dec. 6	
Site	Mean	Std. Dev	Mean	Std. Dev	Mean	Std. Dev	Mean	Std. Dev	Mean	Std. Dev
A	< MDL	-	< MDL	-	1.4	-	< MDL	-	< MDL	-
B	< MDL	-	< MDL	-	< MDL	-	< MDL	-	< MDL	-
C	< MDL	-	< MDL	-	< MDL	-	< MDL	-	< MDL	-
D	< MDL	-	< MDL	-	< MDL	-	< MDL	-	< MDL	-
G24	< MDL	-	7.0	0.9	5.1	1.5	2.4	1.1	9.9	0.9
G25	< MDL	-	5.6	0.3	5.2	0.7	2.4	0.6	9.0	0.4

< MDL indicates less than method detection limit.
Dash indicates no standard deviation available (n < 2).

Lincomycin Temporal Sampling										
	Sept. 30		Oct. 21		Nov. 6		Nov. 19		Dec. 6	
Site	Mean	Std. Dev	Mean	Std. Dev	Mean	Std. Dev	Mean	Std. Dev	Mean	Std. Dev
A	< MDL	-	< MDL	-	< MDL	-	16	7.0	86	5.7
B	< MDL	-	< MDL	-	< MDL	-	78	25	26	22
C	< MDL	-	< MDL	-	< MDL	-	82	14	5.9	1.6
D	< MDL	-	< MDL	-	< MDL	-	< MDL	-	< MDL	-
G24	< MDL	-	< MDL	-	< MDL	-	< MDL	-	< MDL	-
G25	< MDL	-	< MDL	-	< MDL	-	< MDL	-	< MDL	-

< MDL indicates less than method detection limit.
Dash indicates no standard deviation available (n < 2).

Monensin Temporal Sampling										
	Sept. 30		Oct. 21		Nov. 6		Nov. 19		Dec. 6	
Site	Mean	Std. Dev	Mean	Std. Dev	Mean	Std. Dev	Mean	Std. Dev	Mean	Std. Dev
A	< MDL	-	< MDL	-	< MDL	-	< MDL	-	< MDL	-
B	< MDL	-	< MDL	-	< MDL	-	< MDL	-	< MDL	-
C	< MDL	-	< MDL	-	< MDL	-	< MDL	-	< MDL	-
D	< MDL	-	< MDL	-	< MDL	-	< MDL	-	< MDL	-
G24	< MDL	-	< MDL	-	< MDL	-	< MDL	-	< MDL	-
G25	< MDL	-	< MDL	-	< MDL	-	< MDL	-	< MDL	-

< MDL indicates less than method detection limit.
Dash indicates no standard deviation available (n < 2).

Chemical Indicators

Ibuprofen Temporal Sampling										
	Sept. 30		Oct. 21		Nov. 6		Nov. 19		Dec. 6	
Site	Mean	Std. Dev	Mean	Std. Dev	Mean	Std. Dev	Mean	Std. Dev	Mean	Std. Dev
A	< MDL	-	< MDL	-	< MDL	-	6.0	1.1	< MDL	-
B	< MDL	-	< MDL	-	< MDL	-	18	11	< MDL	-
C	< MDL	-	< MDL	-	< MDL	-	5.4	0.6	< MDL	-
D	< MDL	-	< MDL	-	< MDL	-	5.5	-	< MDL	-
G24	< MDL	-	< MDL	-	38	13	16	5.7	39	6.6
G25	< MDL	-	< MDL	-	45	12	19	6.6	< MDL	-

< MDL indicates less than method detection limit.
Dash indicates no standard deviation available (n < 2).

Venlafaxine Temporal Sampling										
	Sept. 30		Oct. 21		Nov. 6		Nov. 19		Dec. 6	
Site	Mean	Std. Dev	Mean	Std. Dev	Mean	Std. Dev	Mean	Std. Dev	Mean	Std. Dev
A	< MDL	-	< MDL	-	< MDL	-	< MDL	-	< MDL	-
B	18	1.7	< MDL	-	2.0	-	< MDL	-	< MDL	-
C	6.1	1.4	1.9	0.3	1.4	-	1.5	-	1.9	1.7
D	2.4	0.2	1.6	0.2	< MDL	-	2.2	0.6	3.2	0.3
G24	39	1.6	35	2.8	17	0.9	11	1.1	29	1.2
G25	36	3.2	26	3.6	19	1.3	9.1	0.8	28	0.2

< MDL indicates less than method detection limit.
Dash indicates no standard deviation available (n < 2).

Atrazine Temporal Sampling										
	Sept. 30		Oct. 21		Nov. 6		Nov. 19		Dec. 6	
Site	Mean	Std. Dev	Mean	Std. Dev	Mean	Std. Dev	Mean	Std. Dev	Mean	Std. Dev
A	41	5.6	32	3.3	16	0.7	12	1.5	20	-
B	36	10	43	34	14	3.1	14	1.7	16	-
C	20	2.4	23	1.6	23	4.2	11	1.1	8.4	0.3
D	28	12	20	1.5	20	0.6	10	0.6	3.9	-
G24	61	30	39	2.9	24	3.4	15	2.3	5.9	1.0
G25	48	16	35	8.1	23	1.5	14	0.8	4.6	0.5

< MDL indicates less than method detection limit.
Dash indicates no standard deviation available (n < 2).

Appendix D: River Water and Wastewater Correlations

River Water ($p < 0.001$)

	Sulfamethazine	Sulfamethoxazole	Trimethoprim	Lincomycin	Monensin	Ibuprofen	Venlafaxine	Atrazine	Total Ammonia	Nitrite	Nitrate	Total Phosphorus	Dissloved Chloride
Sulfamethazine	1.000												
Sulfamethoxazole	0.050	1.000											
Trimethoprim	0.019	0.915	1.000										
Lincomycin	0.028	0.024	-0.010	1.000									
Monensin	N/A	N/A	N/A	N/A	1.000								
Ibuprofen	0.038	0.408	0.628	0.145	N/A	1.000							
Venlafaxine	-0.034	0.799	0.702	-0.072	N/A	0.280	1.000						
Atrazine	0.325	0.298	0.328	0.332	N/A	0.362	0.099	1.000					
Total Ammonia	0.008	0.276	0.492	-0.055	N/A	0.867	0.258	0.230	1.000				
Nitrite	0.007	0.615	0.750	0.134	N/A	0.628	0.433	0.246	0.520	1.000			
Nitrate	0.322	0.412	0.416	0.099	N/A	0.181	0.167	0.343	0.151	0.512	1.000		
Total Phosphorus	0.204	0.150	0.310	-0.028	N/A	0.421	0.045	0.176	0.439	0.323	0.327	1.000	
Dissolved Chloride	0.048	0.584	0.641	0.215	N/A	0.499	0.377	0.283	0.456	0.744	0.723	0.314	1.000

Wastewater ($p < 0.001$)

	Sulfamethazine	Sulfamethoxazole	Trimethoprim	Lincomycin	Monensin	Ibuprofen	Venlafaxine	Atrazine	Total Ammonia	Nitrite	Nitrate	Total Phosphorus	Dissloved Chloride
Sulfamethazine	1.000												
Sulfamethoxazole	-0.268	1.000											
Trimethoprim	0.028	0.743	1.000										
Lincomycin	-0.320	-0.358	-0.467	1.000									
Monensin	N/A	N/A	N/A	N/A	1.000								
Ibuprofen	0.702	-0.004	0.437	-0.248	N/A	1.000							
Venlafaxine	-0.158	0.824	0.723	-0.197	N/A	0.299	1.000						
Atrazine	-0.320	-0.389	-0.489	-0.167	N/A	-0.248	-0.356	1.000					
Total Ammonia	0.916	-0.395	-0.258	-0.242	N/A	0.364	-0.420	-0.242	1.000				
Nitrite	0.680	-0.094	0.389	-0.199	N/A	0.991	0.206	-0.196	0.347	1.000			
Nitrate	-0.877	0.506	0.210	-0.057	N/A	-0.457	0.461	0.418	-0.906	-0.472	1.000		
Total Phosphorus	0.728	-0.008	0.454	-0.313	N/A	0.987	0.217	-0.244	0.407	0.986	-0.496	1.000	
Dissolved Chloride	0.389	0.283	0.571	-0.919	N/A	0.543	0.324	0.165	0.174	0.505	0.033	0.576	1.000